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ROLE OF MONOCARBOXYLATE TRANSPORTERS IN PROSTATE CARCINOMA

Tese de Candidatura ao grau de Doutor em Ciências (Especialidade em: Patologia e Genética Molecular), submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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Pértega-Gomes N, Vizcaíno JR, Miranda-Gonçalves V, Pinheiro C, Silva J, Pereira H, Monteiro P, Henrique RM, Reis RM, Lopes C, Baltazar F. **"Monocarboxylate transporter 4 (MCT4) and CD147 overexpression is associated with poor prognosis in prostate cancer.**" BMC Cancer. 2011 Jul 25;11:312. doi: 10.1186/1471-2407-11-312. (Citações ISI: 19)

Pértega-Gomes N, Vizcaíno JR, Gouveia C, Jerónimo C, Henrique RM, Lopes C, Baltazar F. **"Monocarboxylate transporter 2 (MCT2) as putative biomarker in prostate cancer."** Prostate. 2013 May;73(7):763-9. doi: 10.1002/pros.22620. Epub 2012 Nov 28.

Pértega-Gomes N, Valença I, Vizcaíno JR, Ribeiro D, Baltazar F. **"Localization of MCT2 at peroxisomes is associated with malignant transformation in prostate cancer"** to be published in an international scientific periodical with referees.

Pértega-Gomes N, Vizcaíno JR, Sousa S, Coelho R, Attig J, Jurmeister S, Oliveira E, Pereira L, Pinheiro C, Jerónimo C, Henrique RM, Lobo da Cunha A, Lopes C, Maximo V and Baltazar F. "**Metabolic heterogeneity in prostate cancer is linked to disease progression and aggressiveness"** to be published in an international scientific periodical with referees.

Pértega-Gomes N, Vizcaíno JR,Gouveia C, Lopes C and Baltazar F**. "A lactate shuttle system between tumour and stromal cells is associated with poor prognosis in prostate cancer"** to be published in an international scientific periodical with referees.

Pértega-Gomes N and Baltazar F. **"Explotation of MCTs as potential therapeutic targets in prostate cancer"** invited work to be published in Advances in Prostate Cancer Research and Treatment journal.

TABLE OF CONTENTS

ABBREVIATION LIST

AE - Anion Exchanger

AICAR- 5-amino-4-imidazolecarboxamide riboside

AJCC – American Joint Committee on Cancer

AMPK – Adenosine Monophosphate-activated Protein Kinase

ARE – Androgen Response Elements

ATP – Adenosine Tri-Phosphate

BSG - Basigin

CA - Carbonic Anhydrase

CHC - a-cyano-4-hydroxycinnamate

CREB - cAMP-response element-binding protein

DAB – Diaminobenzidine

DFS – Disease-Free Survival

DIDS - 4,4'-diisothiocyanostilbene-2,2'-disulphonate

EMMPRIN - Extracellular Matrix Metalloproteinase Inducer

FdG-PET - ^{18}F-fluorodeoxyglucose Positron Emission Tomography

FDG – 2-(18F) –Fluoro-2-Deoxy-D-Glucose

GLUT - Glucose Transporter

HK – Hexokinase

HIF - Hypoxia-Inducible Factor

HREs – Hypoxic – Response Elements

LDH - Lactate Dehydrogenase

MCT - Monocarboxylate Transporter

MDR1 – Multidrug Resistance Protein 1

NAD – Nicotinamide Adenine Dinucleotide

NF-kB - Nuclear Factor-kappa B

NHE1 - Na+/H+ exchanger 1

NMP1 - Nuclear Matrix Protein-1

Nrf2 - NF-E2 related factor 2

PBS- Phosphate-Buffer Saline

PBK – Protein Binding Kinase

pCMBS - p-Chloromercuribenzene Sulphonate

PDH - Pruvate Dehydrogenase

PDK1 - Pyruvate Dehydrogenase Kinase 1

PK – Pyruvate Kinase

PKC - Protein Kinase C

PPAR - Peroxisome Proliferator-activated receptor

PPRE - Peroxisome Proliferator-activated receptor response elements

PSA- Prostate Specific Antigen

RP- Radical Prostatectomy

siRNA - Small-interfering RNA

SLC- Solute Carrier

TCA - Tricarboxylic Acid

TGF – Transforming Growth Factor

TMD - Transmembrane Domain

TNF-a - Tumour Necrosis Factor-alpha

TNM- Tumour-Node-Metastasis

USF - Upstream Stimulatory Factor

UTR – Untranslated Region

VEGF - Vascular Endothelial Growth Factor

ABSTRACT

The metabolic adaptation is now considered a new hallmark of cancer, in which cancer cells exhibit high rates of glucose consumption and consequent lactate production. To ensure the rapid efflux of lactate, most of cancer cells express high levels of monocarboxylate transporters (MCTs). It has long been recognized that MCTs might represent good targets for chemotherapy and several *in vitro* studies have shown the potential of this approach, however, regarding prostate cancer the impact of MCTs inhibition and their regulation during prostate cancer progression still largely unknown. Also, it remains unclear what is the predominant metabolic pathway in prostate cancer and therefore which pathway represents the most appropriate target for PCa therapy.

The major aim of this thesis was to investigate MCTs expression and regulation during prostate cancer progression and whether these transporters could represent promising targets for prostate cancer treatment.

MCTs expression together with other key metabolic-related proteins was studied in a large series of human prostate tissues and its clinico-pathological value was assessed. *In vitro* studies were then carried in order to understand how metabolic demands change during prostate cancer progression and how these changes could represent promising targets for prostate cancer disease, with a special emphasis to MCTs in this context.

The obtained results indicate that prostate cancer is not as glycolytic as other tumour types and increased glycolysis is mainly found in a most advanced stage of the disease. Due to this, we observed that different isoforms of MCTs are differentially expressed across prostate cancer progression and accordingly to the demands of prostate cancer cells at each stage. We showed that MCTs are not restricted to the plasma membrane of prostate cells, indicating their involvement in alternative cellular roles different from glycolysis. Finally, we showed that MCTs inhibition affects viability and proliferation of prostate cancer cell lines under different tumour environment conditions.

In conclusion, the results presented in this thesis have an important impact on the comprehension of the metabolic demands of prostate cancer tumours during disease progression in which MCTs play an important role and might

represent promising therapeutic targets in different phases of neoplastic transformation and progression.

RESUMO

O conceito de alterações metabólicas sofridas pelas células tumorais foi recentemente incluído nas principais alterações sofridas no cotexto de transformação maligna. Uma das principais características metabólicas das células tumorais consiste no seu elevado consumo de glucose e consequente produção de lactato.

De modo a assegurar o rápido efluxo de lactato, a maioria das células malignas expressam elevados níveis de transportadores de monocarboxilatos (MCTs). A inibição destes transportadores tem sido apresentada como um bom alvo para quimioterapia e vários estudos *in vitro* demonstraram já o potencial desta abordagem, no entanto, no contexto do carcinoma da próstata o impacto da inibição MCTs assim como a sua regulação durante a progressão tumoral continua em grande parte desconhecida. Adicionalmente, ainda não está claro qual é a via metabólica predominante no cancro da próstata e, portanto, qual a que se apresenta como alvo mais apropriado para a terapia.
O principal objetivo deste trabalho foi investigar a expressão e regulação dos MCTs durante a progressão do carcinoma da próstata e se estes transportadores poderiam representar alvos promissores no tratamento desta doença.
A expressão de MCTs juntamente com outras proteínas relacionadas com o metabolismo foi estudada usando uma grande série de tecidos de próstata humana e o seu valor clínico-patológico foi avaliado. Estudos *in vitro* foram realizados a fim de compreender como variam as exigências metabólicas das células durante a progressão tumoral e de que forma estas mudanças poderiam representar alvos terapêuticos para a doença, dando especial ênfase aos MCTs neste contexto.
Os resultados obtidos indicam que o carcinoma da próstata não é tão glicolítico como outros tipos de tumor e que o aumento da glicólise é principalmente encontrado num estadio mais avançado da doença. Foi observado que diferentes isoformas de MCTs estão diferencialmente expressas em diversas fases de progressão do carcinoma da próstata e potencialmente relacionados com diferentes vias metabólicas e portanto não restritas ao seu clássico papel de transportadores de lactato na membrana citoplasmática e que a inibição das diferentes isoformas em diferentes condições afeta a viabilidade e proliferação

das células malignas. Em conclusão, os resultados apresentados nesta tese são pioneiros na compreensão das exigências metabólicas do carcinoma da próstata durante a progressão tumoral, contextualizando os MCTs como possíveis alvos terapêuticos em diferentes fases da transformação neoplásica e progressão maligna.

AIMS AND THESIS LAYOUT

Metabolic changes during malignant transformation have been noted for many years. Otto Warburg first reported that cancer cells preferentially rely on glycolysis for energy production, even in the presence of oxygen, leading to production of high levels of lactate. The crucial role of lactate efflux and exchange within the tumour microenvironment drew attention to monocarboxylate transporters (MCTs). MCTs have been pointed as promising targets in cancer therapy and described in a large variety of tumours, however, studies showing how these isoforms contribute to the acquisition of the malignant phenotype are scarce and regarding prostate cancer it is totally unknown.

The main aim of this thesis was to characterize MCTs expression in prostate tissues, namely prostate cancer, as well as to give a contribution to understand the role of MCTs in prostate cancer maintenance and progression providing evidence for the exploitation of MCTs as potential targets for prostate cancer therapy.

This thesis is organized in **7 related chapters**, aiming to show the most important results obtained during the development of this scientific project.

In **Chapter 1** a general introduction to the Hallmarks of cancer with a special emphasis to altered cellular metabolism as a new hallmark of cancer and the "Warburg effect" as the basis to explore MCTs as suitable targets for cancer therapy is provided. Also, the current knowledge on prostate cancer metabolism is reviewed in this chapter.

In **Chapters 2-6** major findings about metabolic changes across prostate cancer malignant transformation as well as the value of using MCTs as targets for prostate cancer therapy is presented. These chapters include results already published or submitted for publication in international scientific periodicals with referees, giving a contribution to understand the role of MCTs in the context of prostate cancer. In **Chapter 2** the expression of MCTs in prostate tissues and the clinico-pathological value of this expression is presented. **Chapter 3** explores more deeply the role of MCT2 in prostate cancer metabolism. In **Chapter 4** a panel of key metabolic-related proteins expression was studied in an even bigger casuistic of prostate tissues. *In vitro* assays were also performed showing

important metabolic alterations across prostate cancer malignant transformation and how MCTs inhibition affects prostate cancer cells in different tumour environment condition. **In Chapter 5** an effort to understand the metabolic alterations in the stroma that surrounds prostate tumours is presented. Finally, **Chapter 6** presents an insight on MCTs regulation across prostate cancer progression and in **Chapter 7,** the main conclusions of **Chapters 2-6** are summarized and some important future directions are suggested. Each of these chapters has their own bibliography section.

CHAPTER 1. GENERAL INTRODUCTION

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1.1 Reprogramming of energy metabolism as an emerging Hallmark of Cancer

Advances in cancer research have generated a rich but also extremely complex knowledge revealing cancer as a disease that involves several dynamic changes. In order to organize the complexity of cancer, the biological capabilities acquired during the multistep development of human tumours and shared by all cancers were grouped and denominated as the “hallmarks of cancer”. Sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis were the traits established in order to create an organized principle that provides a logical framework to understand the diversity of neoplastic disease [1]. Lately, new hallmarks have emerged and reprogramming of energy metabolism was considered an emerging hallmark of cancer since it was recognized that the chronic and uncontrolled cell proliferation that represents the essence of neoplastic disease involves also adjustments of energy metabolism in order to fuel cell growth and division [2]. Figure 1 represents the last version of the “Hallmarks of cancer”.

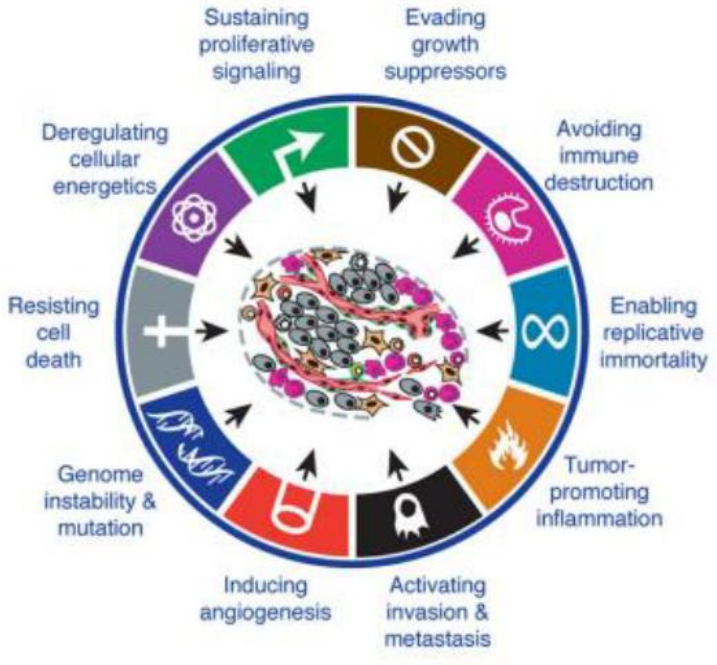

Figure 1. The Hallmarks of Cancer [2].

1.1.1 The Warburg effect as the basis to explore altered cellular metabolism in cancer

Altered energy metabolism is being proved to be as widespread in cancer cells as many of the other cancer-associated traits that have been accepted as hallmarks of cancer. However just recently a major relevance has been given to cancer metabolism, the observation that tumour cells exhibit an altered metabolism when compared to normal cells was made almost one century ago by the Nobel Prize winner Otto Warburg who described it as the first tumour specific-alteration [3]. Warburg described that in contrast to non-malignant cells, many types of cancer cells prefer glycolysis as the ATP source, even in the presence of abundant oxygen. If under aerobic conditions, normal cells process glucose, first to pyruvate via glycolysis in the cytosol and then to carbon dioxide in the mitochondria, under anaerobic conditions, glycolysis is favored and few pyruvate is send to the oxygen-consuming mitochondria. Warburg first observed that cancer cells can reprogram their glucose metabolism, and thus their energy production, by limiting their energy metabolism largely to glycolysis, leading to a state that has been called ‘‘aerobic glycolysis’’ (Figure 2).

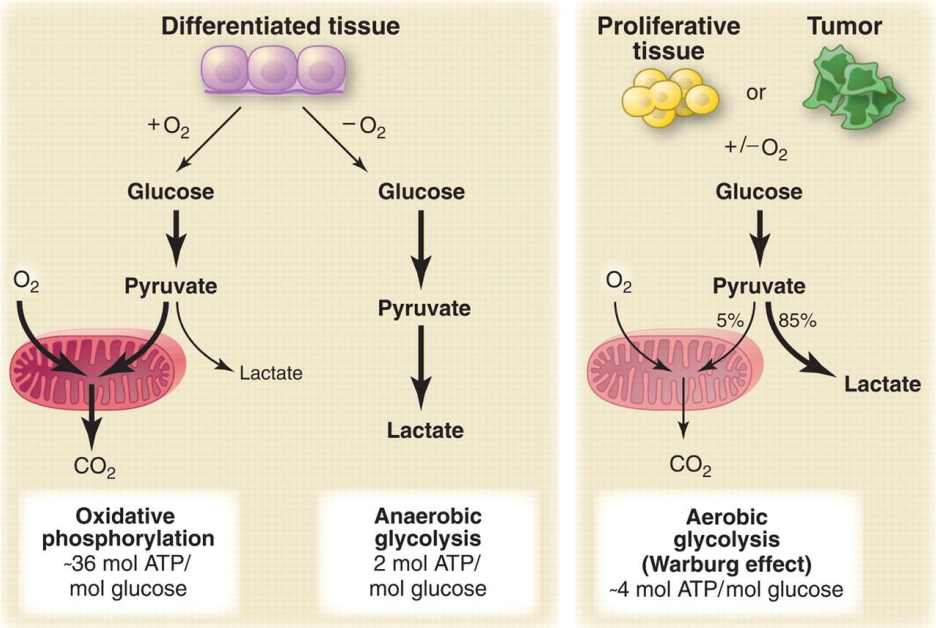

Figure 2. Schematic representation of the differences between OXPHOS, anaerobic glycolysis and “aerobic glycolysis” (Warburg effect) [4].

Glycolysis, the first major pathway of cellular metabolism, occurs in the cytoplasm and is functional even in the absence of oxygen. While glycolysis is able to produce ATP at high rates, it is considered a low efficiency pathway because it produces only two ATP molecules per glucose. In order to compensate for the lower efficiency of ATP production obtained by glycolysis relative to mitochondrial oxidative phosphorylation, cancer cells markedly increased uptake and utilization of glucose in many human tumor types being the rate of entry higher than in normal cells [5-7]. This has proven useful to detect tumours and also monitor their treatment, as it is the basis for the clinical use of positron emission tomography (PET) that uses a radiolabeled analog of glucose (18F-fluorodeoxyglucose, FDG) as a reporter [8]. FDG is recognized as a substrate for glucose transport systems; thus, the rate of entry of this glucose derivate into cells is determined by the activity of glucose transport systems. Inside the cells, the accumulation of the radioactive glucose analogue serves as a read-out for the rate of glucose entry into cells. Since tumour cells exhibit enhanced glucose uptake compared to adjacent normal cells, PET is able to detect tumours and differentiate them from normal tissue [9, 10] (Figure 3).

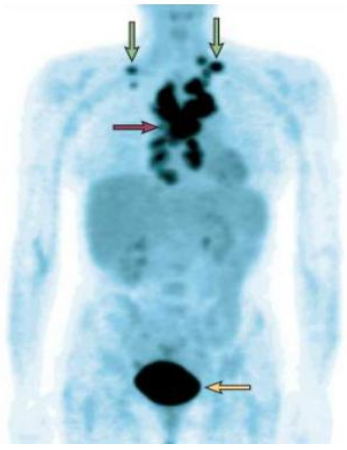

Figure 3. PET imaging with FDG of a patient with lymphoma. The mediastinal nodes (purple arrow) and supravincular nodes (green arrows) show high uptake of FDG, showing that tumours in these nodes have high levels of FDG uptake. The bladder (yellow arrow) also has high activity, because of excretion of the radionuclide [11].

1.1.2 A metabolic switch that confers advantage to cancer cells.

1.1.2.1 Why do cancer cells prefer aerobic glycolysis?

Why a cell would settle for two molecules of ATP per molecule of glucose rather than the additional 34 molecules of ATP from aerobic pathways remains hotly debated. Numerous explanations have been presented including hypoxic selection, mitochondrial dysfunction, AKT activation, and deactivation of the tumour suppressor p53 but none can be generalized to all cell types or types of cancer and a combination of mechanisms are likely to be involved.

The original explanation of Otto Warburg for aerobic glycolysis in cancer cells was irreversible damage of the respiratory chain [3]. Mitochondria are indeed the most important component of the cell and it is known that mitochondrial DNA (mtDNA) mutations and deletions can occur in some contexts where aerobic glycolysis is observed. Alternatively, it is perhaps more likely that the primary metabolic lesion is a deregulation of mitochondrial biogenesis: cancer cells may derive the majority of their ATP from the glycolytic pathway because they do not have a sufficient number of otherwise normal mitochondria to yield ATP from oxidative metabolism. In fact, many cancer cells have fewer mitochondria per cell in comparison to healthy cells [12]. Another hypothesis has come over in the last years, such as the somatic evolution during carcinogenesis that involves changes in the activity of tumour suppressor genes and proto-oncogenes. It is known that some mutations confer a selective advantage to cancer cells, and contribute to malignant transformation. Molecular alterations over the evolution of carcinogenesis are driven by selective pressure within the cellular microenvironment. Early in tumorigenesis, prior to vascularization, the cells in the center of the tumour are unable to access oxygen and nutrients from the bloodstream and experience periods of intermittent hypoxia. Enhanced glycolysis, under such circumstances, may benefit the cell in two ways: 1) by maintaining ATP production when oxygen is limiting, and 2) by producing reducing equivalents that protect the cell from oxidative stress during periods of reoxygenation [13]. However, cancer cells maintain their glycolytic phenotype following vascularization. Therefore, selection

for cancer genotypes that improve glycolysis under anaerobic conditions may result in a genotype that must rely on glycolysis even under aerobic conditions. While these observations are widespread, their molecular underpinnings are complex and not well defined so far.

Several reasons were established in order to explain what drives this glycolytic switch in cancer cells and how it constitutes an advantage for tumour growth.

Some explanations are presented below:

- Tumour cells are able to survive in conditions of fluctuating oxygen tension, condition that would be lethal for cells that rely mostly on OXPHOS to generate ATP [10, 14].
- The acids produced by cancer cells, namely lactic acid, condition their environment, favouring tumour invasion and suppress anticancer immune effectors. Moreover, lactate can be taken up by stromal cells to regenerate pyruvate that either can be extruded to refuel the cancer cell or can be used for OXPHOS. The metabolic netwok established by anaerobic components (cancer cells) and aerobic components (nontransformed stromal cells) allows buffering and recycling products of anaerobic metabolism to sustain cancer cell survival and growth [15-17].
- Tumours are able to metabolize glucose through the pentose phosphate pathway to generate NADPH thus supplying cells' anti-oxidant defenses against hostile microenvironment and chemotherapeutic agents. NADPH can also contribute to fatty acid synthesis [13].
- Cancer cells use intermediates of the glycolytic pathway for anabolic reactions: glucose 6-phosphate for glycogen and ribose 5-phosphate synthesis, dihydroxyacetone phosphate for triacylglyceride and phospholipid synthesis, and pyruvate for alanine and malate sunthesis. Moreover, pyruvate may enter a truncated tricarboxylic acid cycle. The resultant acetyl-CoA is exported from the mitochondrial matrix and becomes available for the synthesis of fatty acids, cholesterol, and isoprenoids [10, 13].
- Reduced ATP generation in mitochondria is a compromise that tumour cells have to make in order to initiate oncogenic transformation. Tumour cells

partially inhibit OXPHOS, thus reducing ATP production, and do not allow electrons to enter in the electron transport chain all the way to oxygen. Consequently, the generation of reactive oxygen species (ROS) increase causing mutations in proto-oncogenes to initiate tumorigenesis [18, 19].

Importantly, recent evidence showed that some tumors have been found to contain distinct subpopulations of cancer cells that differ in their energy-generating pathways. One subpopulation consists of glucose-dependent cells that consume high levels of glucose and secrete high levels of lactate (‘‘Warburg-effect’’), whereas the other subpopulation preferentially utilize the lactate produced by the other cells as their main energy source, employing part of the citric acid cycle to do so. As so, the hypoxic cancer cells rely on glucose for fuel and secrete lactate as waste, this lactate is then imported and preferentially used as fuel by their better-oxygenated cells creating an energetic symbiosis in which lactate is the major player and that represents advantages for the sustaining and progression of the tumours.

Figure 4 shows a model for cell-environment interactions in carcinogenesis. First, it results in a phenotype with a powerful proliferative advantage, in that, through persistent aerobic glycolysis, it is able to alter the local microenvironment in a way that is harmless to itself, but fatal to competing populations. Secondly, acidification of the microenvironment facilitates tumour invasion both through destruction of adjacent normal populations and through acid-induced degradation of the ECM and promotion of angiogenesis [11].

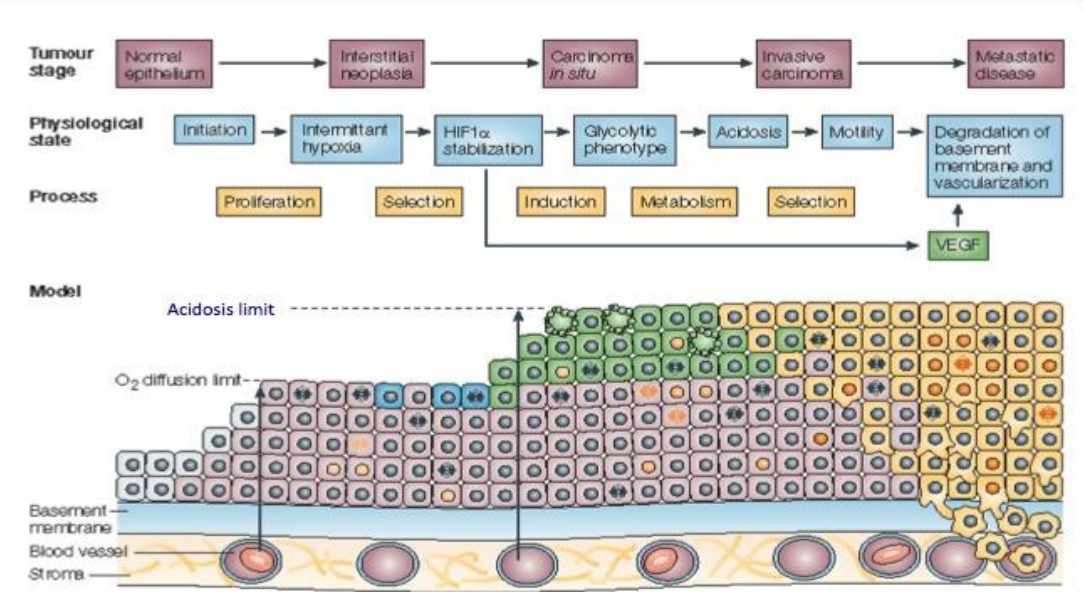

Figure 4. Model for cell-environment interactions in carcinogenesis (from [11]). Cell colours represent different cell types: grey for normal epithelial cells, pink for hyper-proliferative cells, blue for hypoxic cells, green for glycolytic cells and yellow for motile cells. Light orange nuclei represent one mutation while dark orange nuclei represents more than one mutation. Blebbing membranes show apoptotic cells.

1.1.2.2 A deeper insight into Hypoxia and Lactate export: Great contributors to malignancy.

Tumour hypoxia is a common feature of malignancy in solid tumours that is also emerging progressively as a common feature of prostate tumours associated with poor prognosis. The molecular basis of hypoxia in disease progression is well documented, however, the potential role of hypoxia in these processes remains poorly understood.

It is well documented that in response to an adverse microenvironment cancer cells initiate responsive mechanisms that favors cell survival and migration, namely the activation of the transcriptional factor HIF-1alpha, a widely known regulator of hypoxic stress, which has been associated with cancer progression [20-25]. HIF-1alpha subunit is degraded in the presence of oxygen and stabilized under hypoxic conditions (<5%O2) and translocated to the nucleus forming a HIF1alpha/beta complex which can bind to target genes on their hypoxic response elements (HREs) [26, 27]. This biding results in a transcriptional up-regulation of

target genes that mediate multiple adaptations that leads to resistance to hypoxic conditions, namely, genes involved in the glycolytic pathway, as well as the vascular endothelial factor (VEGF) that promotes angiogenesis [10, 28]. Figure 5 shows representative scheme of HIF-1alpha degradation under normoxic conditions and is activation by hypoxia [29].

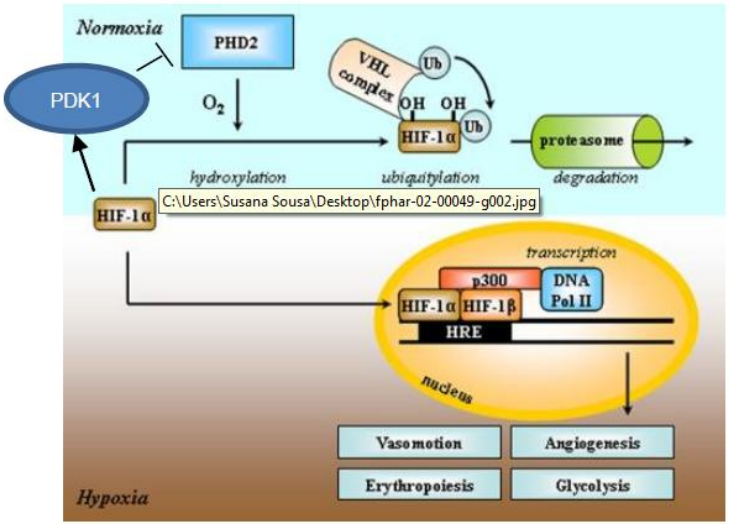

Figure 5. Scheme representing HIF-1alpha degradation under normoxic conitions and is activation by hypoxia. Under normoxia HIF-1alpha is hydroxylated, ubiquitinated and consequently depredated. Under hypoxic conditions HIF-1alpha protein is expressed, migrates to the nucleus and binds to HIF-1beta and further to HRE inpromoting regions, inducing glycolysis, angiogenesis, erytropoiesis and vasomotion [29].

Therefore, adaptation to hypoxia and acidosis will force the emergence of an adaptive phenotype with constitutive up-regulation of glycolysis and resistance to acid-induced toxicity that will invade the normoxic regions and breach the basement membrane towards invasion. Hence, aerobic glycolysis, a common feature in primary tumours, is pointed as a feature required for evolution of invasive human cancers.

Importantly, HIF-1 alpha not only stimulates glycolysis but also decreases oxidative phosphorylation. In a general way, HIF can compromises oxygen consumption by induction of pyruvate dehydrogenase kinase-1 (PDK1) expression, which inhibits the mitochondrial pyruvate dehydrogenase (PDH), preventing conversion of pyruvate into acetyl-CoA and consequently its diversion o the respiratory pathway (Figure 6). As a consequence of this, hypoxic tumour cells are forced as well to increase the glycolytic metabolism to maintain the production

of ATP for cell survival. As so, HIF1-alpha regulates the expression of glucose transporters, namely, GLUT-1 and GLUT-3 to increase glucose uptake and also indces the expression of several other proteins involved in this metabolic cascade, such as, hexokinase II (HK2) and pyruvate kinase type 2 (PKM2). This metabolic reprogramming mediated by HIF1-alpha redirects pyruvate to another HIF1-alpha target, lactate dehydrogenase (LDHA), responsible for the conversion of pyruvate to lactate. Lactate production contributes to acidosis in the tumour microenvironment and therefore to the acid-resistant phenotype leading to an increase in proteins that perform the pH regulation, namely carbonic anhydrase IX (CAIX), which performs the reversible conversion of CO_2 to bicarbonate and proton, contributing to extracellular acidification of tumour microenvironment and consequently to control intracellular pH, being considered a hypoxic marker and a prognostic indicator [27, 30].

Due to all this reasons hypoxia induced metabolic changes represent promising therapeutic targets for cancer therapy with some clinical trials already in course.

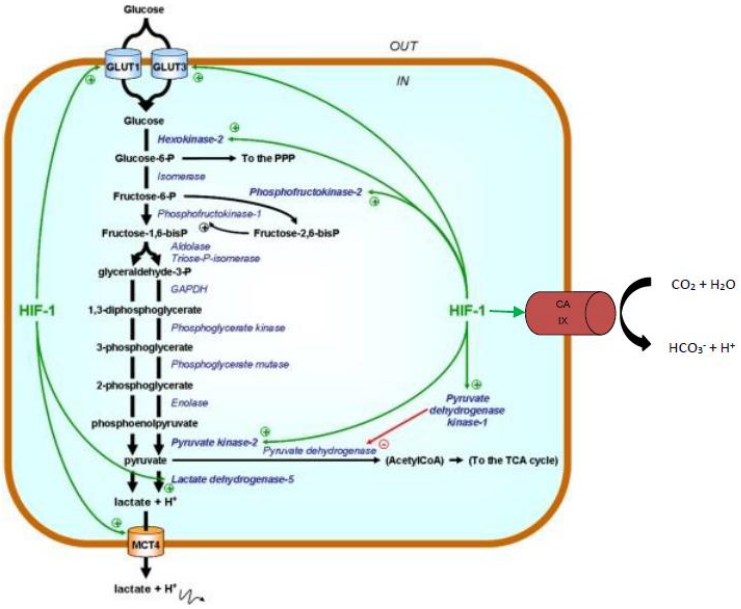

Figure 6. HIF1-alpha expression promotes the expression of various proteins involved in glycolytic metabolism [31].

The lactate produced as an end product of glycolysis is largely associated with poor prognosis, disease-free survival and overall survival in several cancers including prostate cancer [32-34]. Evidence shows that independently of the oxygen condition, lactate can regulate hypoxia inducible genes by stimulating the

accumulation of HIF1-alpha, which would lead in turn to a stimulation of the glycolytic pathway by regulating glycolytic enzymes, providing an important positive feedback in the context of cancer. Moreover, lactate was demonstrated to stimulate molecules involved in the process of cancer invasion and metastasis. CD44, VEGF and transforming growth factor (TGFB2) are some of them [35-39].
Besides glycolysis, it is important to recognize that there are other pathways that can also contribute to lactate production in solid tumours such as glutaminolysis and serinolysis (Figure 7) [40,41].

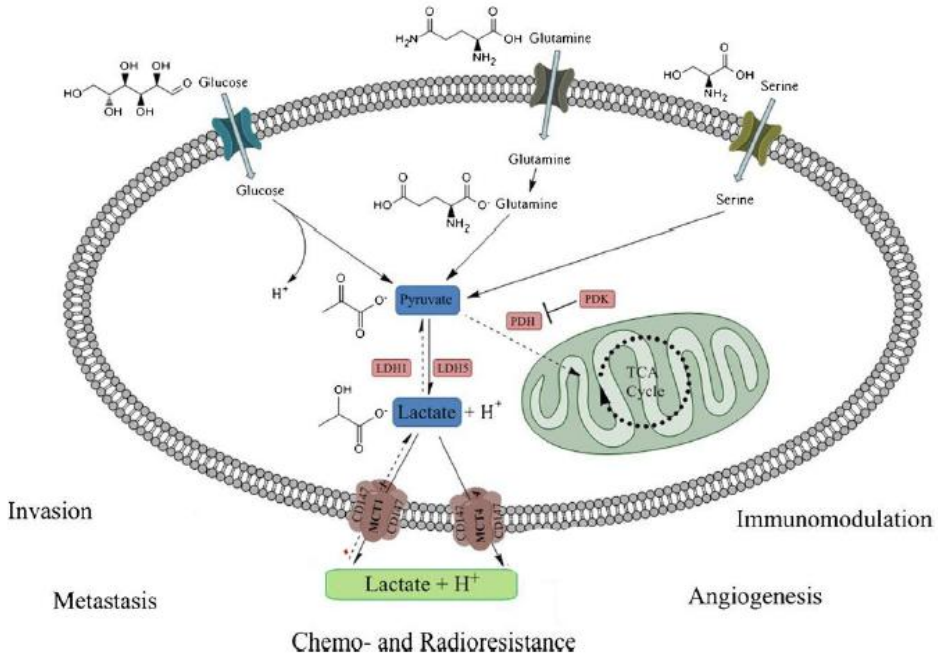

Figure 7. Overview of the metabolic pathways leading to lactate production in cells (continuous lines). The discontinuous arrows indicate lactate uptake and flow inside oxidative cancer cells.

Besides being mainly an end-product, lactate exported might also be a substrate for neighbor cells, a phenomenon known as “cell-cell lactate shuttle”, in which the peripheral and oxygenated oxidative cells consume the lactate produced by the central and hypoxic glycolytic cells (Figure 8).

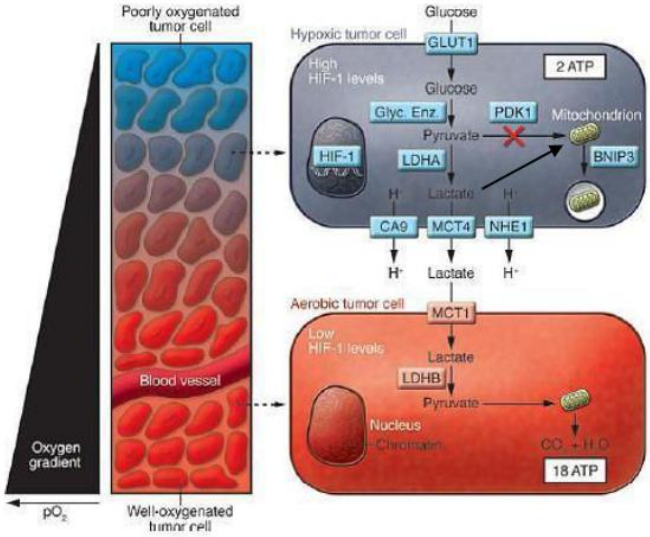

Figure 8. Scheme representing intratumoural hypoxia and metabolic symbiosis.

The crucial role of lactate efflux and exchange within the tumour microenvironment, drew attention to monocarboxylate transporters (MCTs) which transport monocarboxylates such as lactate across the membranes, and therefore play a central role in cellular metabolism and metabolic communication between tissues.

1.2 Monocarboxylate Transporters (MCTs) in the context of cellular metabolism.

The transport of monocarboxylates across the plasma membrane was originally thought to be via non-ionic diffusion of the free acid, however, the demonstration that lactate and pyruvate transport into human erythrocytes could be strongly inhibited after treatment with chemicals, allowed the identification of a specific monocarboxylate transport mechanism. The monocarboxylate transport was then characterized extensively in different cell types and the observed characteristics led to the rationale for of the existence of a family of monocarboxylate transporters [42-43].

1.2.1 The MCT Family

MCTs are encoded by the SLC16 gene family, which is conserved among species, including rat, mouse, chicken and others. The family is presently composed by 14 members, identified through screening of genomic and expressed sequence tag databases [43].

These proteins catalyse the transport of lactate with a proton, with no energy input involved in this process [42, 44].

It is predicted that the topology of MCTs consists of 12 transmembrane domains (TMDs) with the N- and C- termini located in the cytoplasm, as illustrated in Figure 9 for MCT1. The TMDs are highly conserved among isoforms with the greatest sequence variations observed in the C-terminus and the large intracellular loop between TMDs 6 and 7, which has a range of 29-105 amino acid residues [45] . This observed variability is common to transporters with 12 TMDs and it is thought that these sequence variations are related to substrate specificity or regulation of transport activity [44, 45]. Theoretical predictions and experimental evidence indicate that none of the MCT family members is glycosylated [46-49]. To function, an MCT translocates a proton and a monocarboxylate through the plasma membrane by an ordered mechanism in which H+ binding is followed by monocarboxylate binding to the protonated transporter [49, 50]. Therefore, MCT activity is dependent on both besides substrate concentration and proton gradient between the extracellular and intracellular milieus.

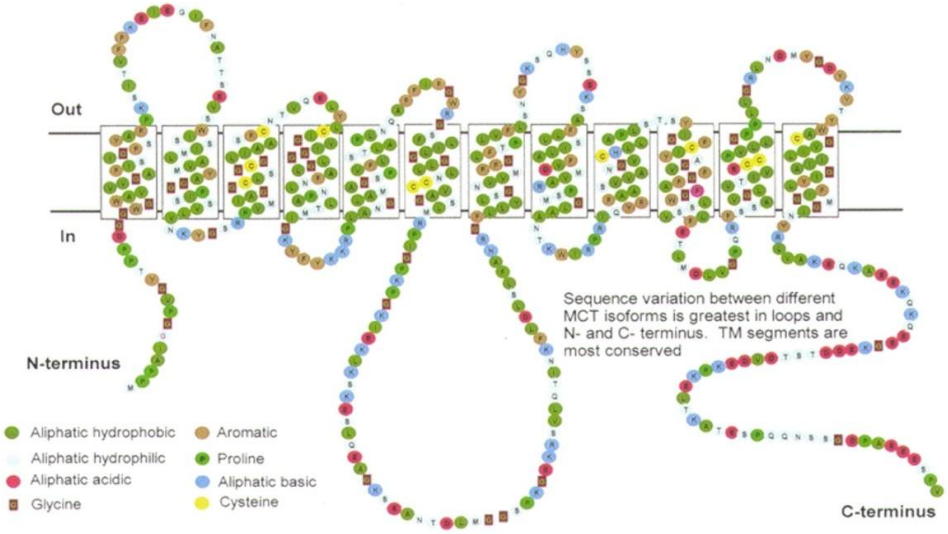

Figure 9. Proposed topology of MCT1. The model shown is that predicted from the primary sequence using hydropathy plots and subsequently confirmed by proteolytic cleavage and labelling experiments as described by Juel & Halestrap [44].

Lactate is indeed the monocarboxylate whose transport across the plasma membrane is quantitatively more important, however, MCTs are also important for the transport of many other metabolically important monocarboxylates such as pyruvate, the branched-chain oxoacids derived from leucine, valine and isoleucine, and the ketone bodies acetoacetate, β-hydroxybutyrate and acetate [45]. Consequently, MCTs have a central role in mammalian cell metabolism and are critical for the communication between cells as illustrated in Figure 10.

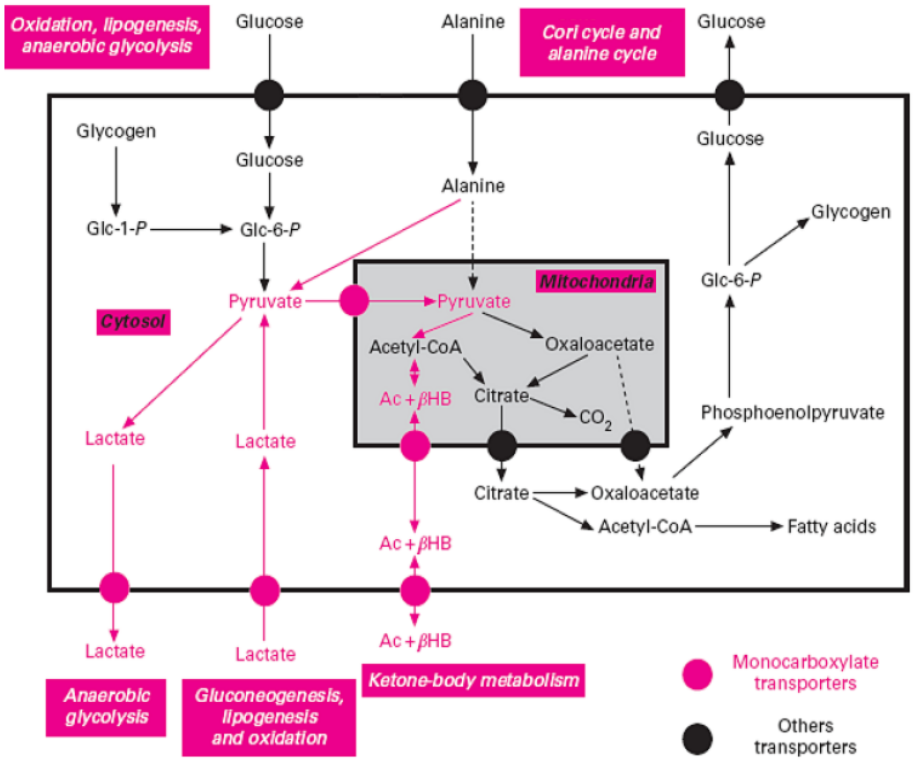

Figure 10. Metabolic pathways involving monocarboxylate transport across the mitochondrial and plasma membranes [45].

Besides being a family of 14 members, only the first four (MCT1-MCT4) have been demonstrated experimentally to facilitate the proton-linked transport of metabolically important monocarboxylates [46-49]. Since MCT3 is a very specialized MCT, being limited to the retinal pigment and choroid plexus epithelia [50,51] this introduction will only focus on MCT1, MCT2 and MCT4 isoforms, whose function is responsible for the name of this family of transporters [52-56].

Figure 11 shows that MCT1-MCT4 are associated in the same cluster presenting high homology. Importantly, this cluster is yet sub-divided in two branches MCT1-2 and MCT3-4 which correlates with the transported substrate as well as the affinities by which those transport is performed.

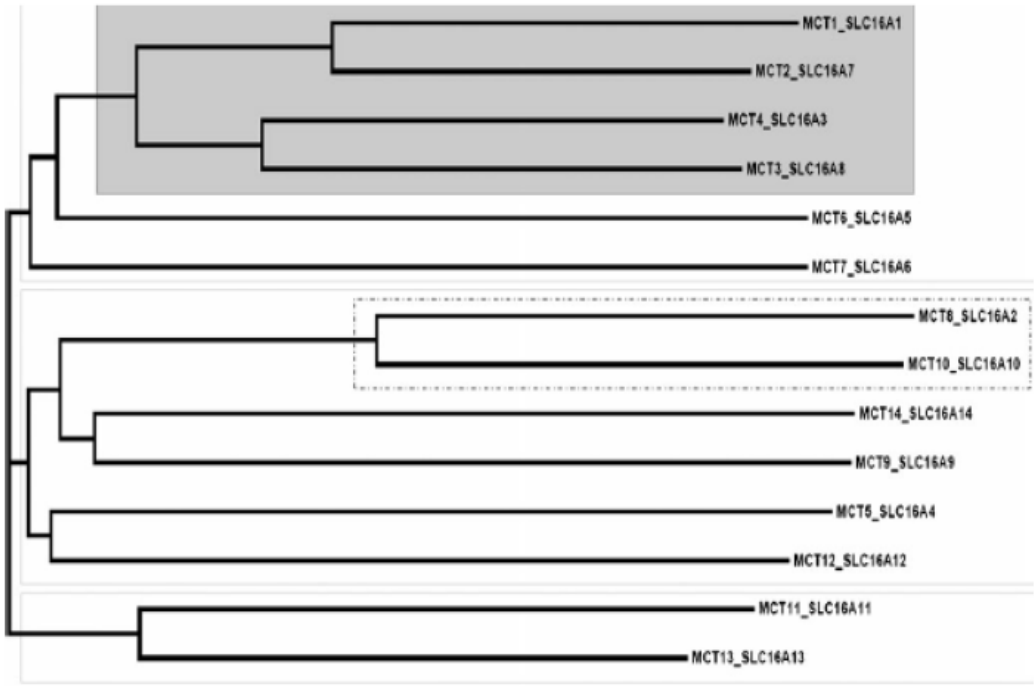

Figure 11. Human MCT family members' phylogram, based on amino acid sequence. Boxes limited by dots represent the main clusters [57].

1.2.1.1 MCT1

Human MCT1 gene, SLC16A1 is located on chromosome 1 (1p13.2-p12) and comprises 5 coding exons. MCT1 functional protein is composed by 500 amino acids and has a molecular weight of 53.958 Daltons [58].

Among the MCT family, MCT1 isoform is the most well studied and functionally characterized member, since it is the only MCT expressed in human erythrocytes. Although ubiquitously expressed, MCT1 is especially prominent in heart and red muscle, where it is up-regulated in response to increased work, suggesting a special role in lactic acid oxidation [59,60]. The wide pattern of expression observed for MCT1 may be explained by its substrate affinities that indicate that

MCT1, may be involved in both uptake and efflux of monocarboxylates from cells. MCT1 transports a variety of substrates including short chain (C2-C5) unbranched aliphatic monocarboxylates such as acetate and propionate. Monocarboxylates with C2 or C3 substitutions (excluding amino- and amido-) are also transported or even preferred (e.g pyruvate, L-lactate, acetoacetate and β- hydroxybutyrate) (35-37c). The simplest monocarboxylate, formate, is a poor substrate whereas bicarbonate; dicarboxylates, tricarboxylates and sulphonates are not transported. Also MCT1 is stereoselective for lactate but not for β-hydroxybutyrate.

MCT1 was also found in mitochondria [61,62] and peroxisomes [63], where it is believed to participate in a lactate oxidation complex to maintain organelle redox and proper functioning.

1.2.1.2 MCT2

MCT2 is encoded by the SLC16A7 gene located in chromosome 12 (12q13) and comprises 5 coding exons [64]. Although only one transcript is identified, there is evidence for alternatively spliced mRNA species in human and rat [51, 63], but no evidence of splice variants of the protein. This isoform shares approximately 50% sequence identity with MCT1, contains 478 amino acids and has a molecular weight of 52,186 Da. The fact that, when both MCT1 and MCT2 are expressed in the same tissue, the expression pattern is cell-specific, suggested a distinct functional role between these isoforms [64-68]. Subsequent expression of MCT2 in Xenopus oocytes revealed its unique biochemical feature of facilitating the proton-linked transport of especially pyruvate, with a considerable high affinity, supporting the previous evidence of an alternative biological role [69]. As so, MCT2 also catalysis the proton-linked transport of a range of monocarboxylates, but with a considerably higher affinity than MCT1.

As a higher affinity transporter, MCT2 has also a more restricted expression, being adapted to perform the uptake of monocarboxylates into cells. As a result, MCT2 is found in tissues that use lactate as a respiratory fuel, like brain or cardiac and skeletal muscle, kidney and liver were lactate is the major gluconeogenic substrate

[66,67,69,70]. As MCT1, MCT2 is also found in mitochondria [71,72] and peroxisomes [63].

1.2.1.3 MCT4

MCT4 is encoded by the human SLC16A3 gene, which is located in chromosome 17 (17q25.3), comprises 5 exons and 3 transcripts, with different initiation sites but no difference in protein product has been identified. The protein is constituted by 465 amino acids, corresponding to a molecular weight of 49,469 Da. MCT4 demonstrates remarkable similarities with MCT1 and in contrast to MCT2 has a broader distribution. The principal difference between MCT4 and MCT1 isoforms lies on their specific localization and substrate affinities. MCT4 shows a much lower affinity for substrates than MCT1 and MCT2. Accordingly, MCT4 is predominantly expressed in glycolytic cells such as white muscle and white blood cells, suggesting that its physiological function is lactate efflux and in fact, the kinetic properties of MCT4 show that this isoform is adapted to the export of lactate. This led to the hypothesis that MCT4 might be of particular importance in cells that rely on high rates of glycolytic metabolism to meet their energy demands, producing high amounts of lactate that need to be rapidly exported, such as cancer cells [73].

1.2.1.4 Other MCT isoforms

Other MCT isoforms are being characterized in the last years. SLC18A2 (MCT8) gene has been described as a thyroid hormone transporter [74] and mutations in this gene have been associated with X linked severe mental retardation and neurological dysfunction [76-80]. MCT6 (SLC16A5) transports bumetanide, but neither L-lactic acid or L-tryptophan, in a pH- and membrane potential-sensitive but in a non-proton gradient-dependent manner [81]. MCT9 (SLC16A9) polymorphism was found to be associated with altered serum uric acid [82] however MCT9 substrate is still unknown. Importantly, SLC16A12 (MCT12) has been identified as a possible biomarker for colon, prostate and breast carcinoma due to gene hypermethylation [83] however the substrate for MCT12 is still

unknown, as for the remaining members of the family (MCT5, MCT7, MCT11, MCT13 and MCT14).

Table 1 summarizes the characteristics of the human SLC16 family of transporters and Table 2 shows the substrate affinities for the various MCT isoforms in humans and rats.

Table 1. The human SLC16 family of transporters [47].

MCT	UniGene name	Alternate (*former) Name	Sequence accession ID	Human gene locus	Tissue distribution	Transport mechanism
MCT1	SLC16A1		NM_003051	1p13.2	Ubiquitous	H^+ cotransporter exchanger
MCT2	SLC16A7		NM_004731	12q14.1	Testis, liver, kidney, skeletal muscle, heart, brain, spleen, pancreas	H^+ cotransporter
MCT3	SLC16A8	REMP	NM_013356	22q13.1	Retinal pigment epithelium (RPE), choroids plexus, aorta, placenta, kidney	H^+ cotransporter
MCT4	SLC16A3	(*MCT3)	NM_004207	17q25.3	White muscle, white blood cells, tumors, RPE, brain kidney, placenta, small intestine, lung, heart	H^+ cotransporter
MCT5	SLC16A4	(*MCT4)	NM_004696	1p13.3	Placenta, intestine, colon	Orphan
MCT6	SLC16A5	(*MCT5)	NM_004695	17q25.1	Kidney, muscle, placenta, intestine, brain, heart, pancreas, prostate, lung	Facilitated diffusion
MCT7	SLC16A6	(*MCT6)	NM_004694	17q24.2	Pancreas, brain, muscle	Orphan
MCT8	SLC16A2	XPCT (*MCT7)	NM_006517	Xq13.2	Liver, brain, kidney, heart, placenta	Orphan
MCT9	SLC16A9		BN000144	10q21.2	Endometrium, testis, ovary, breast, brain, kidney, adrenal, retina	Orphan
MCT10	SLC16A10	TAT1	NM_018593	6q21-q22	Intestine, kidney, skeletal muscle, heart, liver, placenta	Facilitated diffusion/ exchanger
MCT11	SLC16A11		NM_153357	17p13.2	Skin, lung, ovary, breast, pancreas, RPE, choroid plexus	Orphan
MCT12	SLC16A12		ENSG000 00152779	10q23.3	Kidney	Orphan
MCT13	SLC16A13		BN000145	17p13.1	Breast, bone marrow	Orphan
MCT14	SLC16A14		BN000146	2q36.3	Brain, heart, ovary, breast, lung, pancreas, RPE, choroid plexus	Orphan

Table 2. Comparision of substrate affinities for the various MCT isoforms in human and rat [47].

Species	Isoform	Expression System	Substrate	Km (mM)
Human	MCT1	*Xenopus* oocytes	Lactate	3.5–6
			Pyruvate	1.8–2.5
			Acetoacetate	5.5
			α-Ketoisovalerate	1.3
			α-oxoisohexanoate	0.67
			α-oxoisovalerate	1.25
			Butyrate	9
			XP13512	0.22
	MCT2	*Xenopus* oocytes	Pyruvate	0.025
	MCT3	ARPE-19 cells	Lactate	n.a.
	MCT4	*Xenopus* oocytes	L-lactate	28
			D-lactate	519
			Pyruvate	153
			D-β-hydroxybutyrate	130
			Acetoacetate	216
			α-ketobutyrate	57
			α-ketoisocaproate	95
			α-ketoisovalerate	113
	MCT6	*Xenopus* oocytes	Bumetanide	0.084
			Nateglinide	n.a.
			Prostaglandin $F_{2\alpha}$	n.a.
	MCT8	COS1 and JEG3 cells	T_3	n.a.
			T_4	n.a.
Rat	MCT1	*Xenopus* oocytes	Lactate	3.5
			GHB	4.6
		MDA-MB231	γ-hydroxybutyrate	4.6
	MCT2	*Xenopus* oocytes	Lactate	0.74
			Pyruvate	n.a.
	MCT4	*Xenopus* oocytes	L-lactate	34
			Pyruvate	36.3
			2-oxoisohexanoate	13
			Acetoacetate	31
			β-hydroxybutyrate	65
	MCT8	*Xenopus* oocytes	T_3	n.a.
			T_4	n.a.
	MCT10	*Xenopus* oocytes	L-Tryptophan	3.8
			L-Tyrosine	2.6
			L-Phenylalanine	7.0
			L-DOPA	6.4

1.2.2 MCT Regulation

Although the regulatory mechanisms of MCT expression are far from being completely understood, evidence indicates that MCTs might be regulated at various points up to the functional protein. This includes both transcriptional and post-transcriptional level [84-87] that affects protein amounts as well as regulators of transporter activity, like chaperone proteins. Hormone regulation has also been described for MCTs, as well as regulation by signaling pathways like insulin-like growth factor receptor type I (IGF-IR) activation which up- regulates MCT1 [88].

MCT1 expression is particularly more described than the other isoforms in different physiological and pathological conditions. Reports including MCT up-regulation in skeletal muscle in response to training [85, 89-96], downregulation after muscle

denervation and inflammatory bowel diseases [97,98], changes in MCT expression during development [99-103] or substrate induced MCT1 up-regulation [86, 87, 104] are relatively frequent. However and importantly, regulatory mechanisms vary among MCTs isoforms, which allows induction of specific isoforms upon different stimuli, adapting cells to different energy demands.

1.2.2.1 Transcriptional Level

The analysis of SLC16A1 5'-flanking region allowed the identification of putative binding site sequences for the transcription factors USF, nuclear factor-kappaB (NF-kB), activated protein 1 and 2 (AP1 and AP2) and stimulating protein-1 (Sp1). USF1 and USF2 have been described as potential repressor proteins for MCT1 [105] whereas NF-kB pathway has been involved in the butyrate-induced MCT1 up-regulation [106]. AP2 has been associated to protein kinase C (PKC)-dependent stimulation of the SLC16A1 promoter [107] .

Lactate-induced increase in MCT1 has been linked to activation of NF-kB and nuclear factor erythroid 2 (NF-E2) pathways, as well as cAMP-response element-binding protein (CREB) and NF-E2 related factor 2 (Nrf2) transcription factors, the last three elements possessing also putative transcription binding sites in the SLC16A1 5'-flanking region.

The co-activators peroxisome proliferator-activated receptor gamma, co-activator 1 alpha (PGC-1a) [108] and peroxisome proliferator-activated receptor alpha (PPARα) [109,110] have been associated with MCT1, but not with MCT2 and MCT4 up-regulations. Promoter analyses of MCT1, MCT2 and MCT4 have shown that the SLC16A1 promoter contains two peroxisome proliferator-activated receptor response elements (PPRE) while SLC16A7 and SLC16A3 each contain one PPRE.

In expression studies, SLC16A1 was also shown to be activated by c-myc and n-myc proto-oncogenes, while the pro-inflammatory cytokines and TNF-a, have also been implicated in the transcriptional control of MCT1, by down-regulating SLC16A1 transcription [111-113].

Hypoxic conditions, which are known to induce the glycolytic phenotype, are also associated with altered expression of MCTs [114-120]. The first report described a tissue-specific change in MCT expression after chronic hypoxia, where MCT1 did not change in heart, soleus, or gastrocnemius muscles, and MCT4 increased significantly in heart muscle. However, in the plantaris muscle, both MCT1 and MCT4 showed a significant decrease after chronic hypoxia [114]. A subsequent study suggested that the increase in neuronal, astrocytic and endothelial MCT1 expression, observed after permanent occlusion of the left middle cerebral artery, is mediated by the hypoxia-inducible factor-1alpha (HIF-1a) [115], the major transcriptional regulator of adaptation to hypoxic stress; however, this view was promptly contested by other authors, that showed that only MCT4 promoter was activated by hypoxia and that this response was mediated by HIF-1a [116] . Additionally, MCT4, but not MCT1, was shown to be up-regulated by hypoxia in human bladder cancer cells [116] and in trophoblast cells [117], and MCT1 and hypoxia were described as being mutually exclusive [118] . In contrast, recent evidence shows a hypoxia-mediated increase in both MCT1 and MCT4 and decrease in MCT2, with MCT1 and MCT4 change being HIF-1alpha-dependent [119]. As so, MCT1 regulation by hypoxia is still very controversial and more efforts have to be made to understand MCTs' regulation by hypoxia.

1.2.2.2 Post- Transcriptional Level

The relatively long 3'UTR of SLC16A1 (1.6kb), on which initiation factors and regulatory proteins interact to enhance or repress translation [120], suggests that MCT1 expression might also depend on translational regulation in contrast to MCT2 and MCT4 which have much shorter 3'UTRs.

MicroRNAs (miRNAs) are a group of small non-coding RNAs (approximately 22 nucleotides) that play a critical role in a variety of biological processes, like development, differentiation and apoptosis. Mature miRNAs negatively regulate their targets through complementary sequence pairing with the 3' UTR of mRNA targets, inducing transcript degradation or translational repression. One of the most well characterized miRNAs in mammalian nervous system is miR-124, which

has been described to regulate MCT1; miR-124 regulates SLC16A1 through binding to its 3' UTR and MCT1 protein level is reduced after miR-124 transfection [121].

1.2.2.3 Transporter Activity Level

Besides being regulated at transcriptional, translational and post-transcriptional levels, MCTs are also regulated by interactions with other proteins in order to be properly expressed in the cellular membrane. MCT1, and MCT4 are regulated by its association with the cell surface glycoprotein CD147 (also known as basigin or EMMPRIN), while gp70 (EMBIGIN) is described for translocation of MCT2 to the plasma membrane [122].

CD147 does not exclusively act as a chaperone, in fact, it is a broadly distributed plasma membrane glycoprotein, which belongs to the immunoglobulin superfamily. CD147 is ubiquitously expressed on the cell surface, with the highest levels found in metabolically active cells [123-125].

Recently, CD44, a widely distributed transmembrane glycoprotein, and its main ligand, hyaluronan, were implicated in the regulation of lactate efflux and membrane localization of MCTs, in human breast carcinoma cells [126]. This protein co-immunoprecipitates with MCT1, MCT4 and CD147 and co-localizes with these proteins in the plasma membrane. Figure 12 shows a sum of the most significant network found using MetaCore programme for MCT1 (SLC16A1), MCT2 (SLC16A7) and MCT4 (SLC16A3). The figure shows interactions among SLC16A1, SLC16A7 and SLC16A3 and important proteins already mentioned above. Note that these proteins do not act in isolation, but the MetaCore graphic shows the essential elements of the network.

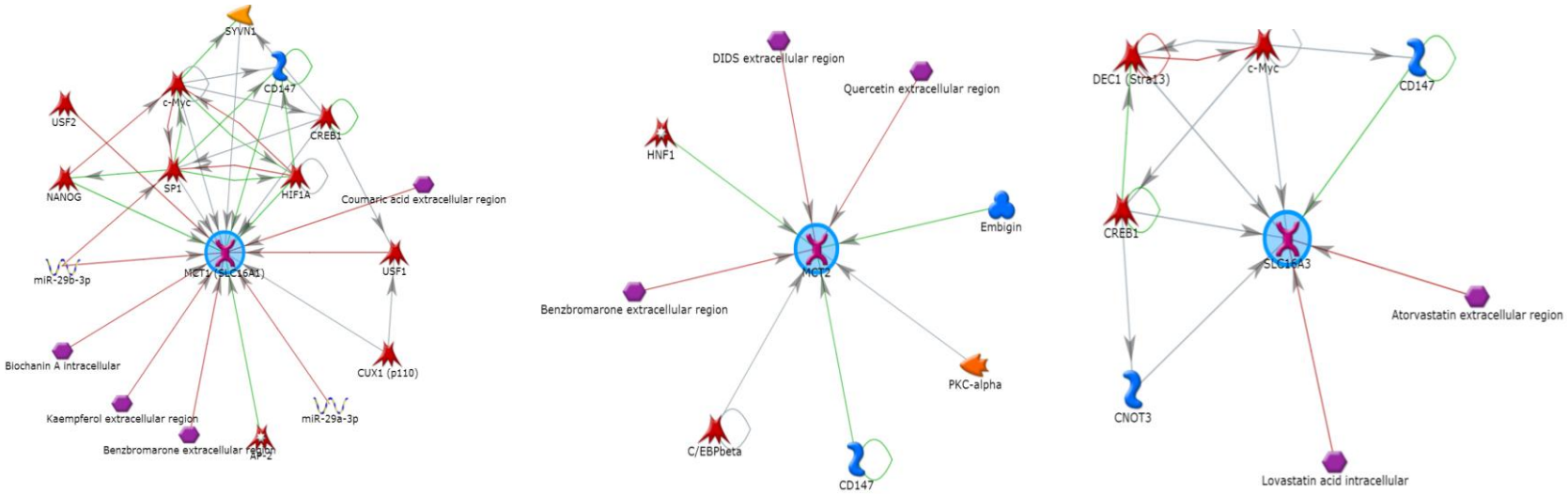

Figure 12. Top scoring MetaCore network including SLC16A1, SLC16A7 and SLC16A3. This gene network is the best fit found by Metacore to represent the relation existent between MCTs and other relevant elements from this database.

1.2.2.4 Hormonal Regulation

MCTs regulation by hormones has been firstly described in 2002, as luminal leptin was shown to significantly up-regulate MCT1-mediated butyrate uptake, in Caco2-BBE cells (human epithelial colorectal adenocarcinoma cells). This increased uptake was achieved through two distinct mechanisms: an increase in the intracellular pool of MCT1 protein, with no changes in CD147 amounts, and translocation of MCT1/CD147 to the apical membrane of Caco2-BBE cell monolayers [127]. Shortly after, the hormones thyroid-stimulating hormone (TSH), noradrenaline, triiodothryonine (T3) and somatostatin were also described as modulators of MCT expression [128-131]. TSH regulates MCT1 protein expression in rat thyroid cells, increasing SLC16A1 transcription, and also increases CD147 protein levels; noradrenaline induces MCT2, but not MCT1 expression, in mouse neurons, at the translational level, with the requirement of an yet unknown transcriptional step; MCT4, but not MCT1, is induced by T3 in rat skeletal muscle; and somatostatin increases MCT1 association with CD147 at the plasma membrane, with an increase in the apical membrane levels of MCT1 protein in parallel to a decrease in the intracellular MCT1 pool.

1.2.3 MCTs inhibition

As previously mentioned, MCTs are transmembrane proteins exposed to the extracellular environment. This feature allows targeting these transporters by systemic application of small-molecule inhibitors. Several agents are known to inhibit MCT activity, in an isoform-dependent manner, including classical inhibitors like α-cyano-4-hydroxycinnamate (CHC), inhibitors that influence MCT activity in a specific manner (eg, MCT1/MCT2 AstraZeneca inhibitors), and compounds that, depending on the context of use, have also been described as inhibitors of MCT activity like cholesterol synthesis inhibitors (statins) and non-steroidal anti-inflammatory drugs.

MCT inhibitors fall into three broad categories:

i) Bulky or aromatic monocarboxylates are competitive inhibitors, including 2-oxo-4-methylpentanoate, phenyl-pyruvate and derivatives of a-cyanocinnamate such as CHC. Also, CHC inhibits the mitochondrial pyruvate transporter as well as the anion exchanger 1 (AE1).
ii) Amphiphilic compounds with widely divergent structures like bioflavonoids (e.g. quercetin and phloretin) and anion transport inhibitors such as 5-nitro-2-(3-phenyl-propylamino)-benzoate and niflumic acid.
iii) Stilbenedisulphonates (eg, 4,4'-diisothiocyanostilbene-2,2'-disulphonate (DIDS) and 4,4'-dibenzamidostilbene-2,20-disulphonate act as reversible inhibitors of MCT1, although with much lower affinity than for AE1. Moreover, miscellaneous inhibitors including thiol reagents, such as the organomercurial thiol reagent p-chloromercuribenzene sulphonate (pCMBS) and amino reagents (eg, pyridoxal phosphate and phenylglyoxal) irreversibly inhibit MCTs.
It is important to emphasize that the affinity of the previous mentioned inhibitors depends on the MCT isoform.

1.2.3.1 The classical MCT inhibitor : CHC

Halestrap and colleagues first described an inhibitory effect of CHC in pyruvate transport across both the plasma membrane of erythrocytes and also the inner membrane of rat liver mitochondria. However, despite being described as an inhibitor of pyruvate entry into mitochondria, internalization of CHC into the cells is still a matter of debate.
Recently, Sonveaux et al. [118] demonstrated that the MCT1 activity blockage with CHC has antitumor effects: daily CHC delivery in mice did not reveal any overt systemic toxicity; Using two experimental mouse models based on MCT1 expression, Lewis lung carcinoma (with MCT1 plasma membrane expression) and hepatocarcinoma (without MCT1 plasma membrane expression), the authors observed a tumour growth retardation when targeting MCT1 with CHC and that the antitumor efficacy of MCT1 inhibition was restricted to tumour cells expressing MCT1 at the plasma membrane. Importantly, inhibiting MCT1 activity with CHC,

the authors observed an overall benefit when combining the previous approach with radiotherapy [118].

1.2.3.2 Specific MCT Inhibitors

It is important to state that neither of the previously mentioned MCT inhibitors is either MCT specific or MCT isoform specific. Consequently, the functional role of MCTs in cells can only be studied using specific inhibitors.

Recently, AstraZeneca divulged a new set of small molecular weight compounds as potent and selective inhibitors of MCT1 in activated human T cells. Upon T-lymphocyte activation, MCT1 expression is rapidly unregulated in order to meet the demand for lactate efflux arising from increased glycolytic rates. Consequently, an effective and highly specific obstruction of MCT1 results in the accumulation of lactate within the cells and feedback inhibition of glycolysis. This metabolic inhibition results in the inability of T-lymphocytes to sustain the rapid rate of cell division during the early immune response. Inhibition of MCT1 with these compounds represents a novel mechanism of immunosuppression distinct from current therapies [132, 133].

1.2.3.3 Other MCT inhibitors

Diets rich in fruits and vegetables were shown by epidemiological studies to be inversely correlated with cancer incidence [134] , playing a pivotal role both in the development and prevention of colon cancer.

Quercetin (3,5,7,3',4'-pentahydroxylflavone) possesses several beneficial biological activities including antioxidant, anti-inflammatory, anti-atherosclerosis and antitumor properties [135-137]. Concerning the antitumor properties in CRC cancer, Quercetin presents antimetastatic potential against melanoma and prostate cancers [138, 139], and suppresses tube formation in human umbilical vascular endothelial cells in response to antiangiogenic activity [140]. Entry of Quercetin into the cells is described to be via GLUT1, 3 and 4 [141-143]. However, it also acts as a high affinity inhibitor of dehydroascorbate and glucose transport

through the same transporters. Quercetin is also described as an inhibitor of the monocarboxylate transporter isoform 1 (MCT1). In previous studies, it was demonstrated that Quercetin acts as reversible noncovalent inhibitor of L-lactate transport by tumour cells or red blood cells [144, 145]. More recently, a study aiming to clarify the role of flavonoids in the modulation of MCT1-mediated transport of hydroxybutyrate *in vitro* and *in vivo* demonstrated that Quercetin (among others) is an effective inhibitor of MCT1-mediated transport [146].

Lonidamine is a derivative of indazole-3-carboxylic acid, which for a long time, has been known to inhibit glycolysis in cancer cells. Although this action was originally attributed to hexokinase inhibition [147, 148], further studies revealed that lonidamine inhibits lactate efflux from cancer cells through inhibition of MCT1 and MCT4 [149, 150]. Actually, despite a lack of knowledge of its precise mechanism of action, lonidamine has been effective in clinical trials against various tumours, especially as a sensitizer to other chemotherapies [151].

1.3 Monocarboxylate Transporters in the Context of Cancer

1.3.1 MCTs in human cancer. What is described?

Regarding cancer research, there are already several evidence for the upregulation of MCTs in several solid tumours, such as colorectal carcinomas [152], uterine cervix carcinomas [153], glioblastomas, breast carcinomas [154, 155] and lung tumours [156], pointing to an important role of this transporters in the maintenance of these malignancies. The first report on MCT expression in human tumor samples described a decrease of MCT1 expression in the colonic transition from normality to malignancy [157], which was further supported by a larger study [158]. In breast cancer, silencing of SLC16A1 by gene promoter hypermethylation was suggested in 4 of 20 breast cancer cases (20 %). In contrast, results from our group showed a significant increase of MCT1 cytoplasmic and plasma membrane expression in breast carcinoma, when comparing to normal breast epithelium [154]. MCT4 only showed a significant increase in tumor samples for cytoplasm expression, with no differences in plasma membrane expression. The literature is also controversial in lung cancer. A study

by Koukourakis and collaborators, no expression of MCTs in normal lung was found, while expression of MCT1 was found in all tumors examined and both MCT2 and MCT4 were also expressed in cancer cells. This study also analyzed the possible metabolic cooperation between lung cancer cells and the tumour-associated stroma, however, tumour associated stroma expressed MCTs weakly [15]. In opposition, a recent study by our group showed that normal lung presents a high frequency of MCT expression and, in fact, MCT4 is less expressed in tumor samples than in normal epithelium.

MCT expression has also been described in some gynecological tumors like cervical and ovarian cancer [159]. In cervical cancer, a significant increase in overall and plasma membrane expression of MCT1 and MCT4 was observed. In ovarian cancer, staining for MCT1 and MCT4 as well as their chaperone CD147 was not found in normal ovarian tissues and benign ovarian tissues, while around 80 % of epithelial ovarian primary and metastatic tumors showed expression of these proteins. MCT1 was significantly associated with low grade tumors, high FIGO stage, presence of residual tumor, lack of relapse and presence of ascites; MCT4 was significantly associated with high grade tumors, high FIGO stage, presence of residual tumor, relapse and presence of ascites. Importantly, MCT expression was associated with the expression of the multidrug resistance markers MDR1 and MRP2. In contrast to what was found in the previous types of tumours, neither MCT1 nor MCT4 were found to be up-regulated in gastric adenocarcinomas [160]. Actually, MCT4 expression was more frequently observed in normal gastric mucosa than in gastric cancer cells and even less frequently observed in lymph-node metastasis, indicating a progressive loss of this MCT isoform with disease progression. Table 3 shows an overview on MCT1 and MCT4 expression and the impact on prognosis in different tumour types.

Table 3. Overview of MCT1 and MCT4 expression and impact on prognosis in different tumour types [57].

MCT1 expression	MCT4 expression	MCT1 expression	MCT4 expression
Colon		**Gynecologic tract**	
↓ from normality to malignancy (Ritzhaupt et al. 1998; Lambert et al. 2002)	Not detected in either normal or tumor tissues (Lambert et al. 2002)	↑ from preinvasive to invasive cervical cancer/associated with metastases in AC (when co-expressed with CD147) (Pinheiro et al. 2008b)	↑ from preinvasive to invasive cervical cancer/↑ AC (Pinheiro et al. 2008b)
(+) in tumor cells but (−) normal epithelium (Koukourakis et al. 2006)	Cytoplasm of cancer cells (Koukourakis et al. 2006)		
↑ in tumor cells, compared to normal epithelium/associated with vascular invasion (Pinheiro et al. 2008a)	↑ in tumor cells, compared to normal epithelium (Pinheiro et al. 2008a)	(+) in ovarian tumor cells (Pinheiro et al. 2010a; Chen et al. 2010), but (−) in normal and benign epithelium (Chen et al. 2010)/associated with low grade, high FIGO stage, residual tumor, lack of tumor relapse and presence of ascites (Chen et al. 2010)	(+) in ovarian tumor cells (Pinheiro et al. 2010a; Chen et al. 2010), but (−) in normal and benign epithelium (Chen et al. 2010)/associated with high grade, high FIGO stage, residual tumor, tumor relapse and presence of ascites (Chen et al. 2010)
(+) in tumor cells (Pinheiro et al. 2010a)	(+) in tumor cells (Pinheiro et al. 2010a)		
Stomach		**Prostate**	
(+) with no change along progression/associated with advanced gastric cancer, Lauren's intestinal type, stage III+IV and lymph-node metastases when (co-expressed with CD147) (Pinheiro et al. 2009b)	↓ from normal tissue, to primary tumor, to lymph-node metastases/associated with early gastric cancer and Lauren's intestinal type (Pinheiro et al. 2009b)	(+) in tumor cells but (−) normal epithelium and PIN lesions/associated with high pretreatment PSA, high Gleason score, high pathological grade and nodal involvement (Hao et al. 2010)	(+) in tumor cells but (−) normal epithelium and PIN lesions/associated with high pretreatment PSA, high Gleason score, high pathological grade and nodal involvement (Hao et al. 2010)
Breast		↓ in tumor cells, compared to normal epithelium/associated with high PSA, absence of perineural invasion and presence of biochemical recurrence (Pertega-Gomes et al. 2011)	↑ in tumor cells, compared to normal epithelium/high PSA levels, advanced tumor stage, higher Gleason score, presence of perineural invasion, and presence of biochemical recurrence (Pertega-Gomes et al. 2011)
↓ due to gene hypermethylation (Asada et al. 2003)	Tendency to be ↑ in tumor cells, compared to normal epithelium (Pinheiro et al. 2010b)		
↑ in tumor cells, compared to normal epithelium/associated with basal-like subtype, high histological grade, estrogen and progesterone receptors, cytokeratins 5 and 14 and vimentin (alone or co-expressed with CD147) (Pinheiro et al. 2010b)	↑ in tumor cells, compared to normal epithelium (Pinheiro et al. 2010a)	**Central nervous system**	
		Strongest in high grade glial neoplasms, compared to low grade glial neoplasms (Froberg et al. 2001)	(-) in glioblastoma (Mathupala et al. 2004)
Lung		(+) in glioblastoma and (−) in normal tissue (Mathupala et al. 2004)	
Cytoplasmic accumulation in alveolar soft-part sarcoma (Ladanyi et al. 2002)	(+) in tumor cells but (−) normal epithelium (Koukourakis et al. 2007)	(+) in neuroblastoma/associated with age >1 year at diagnosis, stage 4 disease, unfavorable Shimada histopathology, DNA diploid index, *n-myc* amplification and high-risk clinical group (COG criteria) (Fang et al. 2006)	
(+) in tumor cells but (−) normal epithelium (Koukourakis et al. 2007)	↓ in tumor cells, compared to normal epithelium (Pinheiro et al. 2010a)		
(+) in tumor cells and normal epithelium (Pinheiro et al. 2010a)			

↓ downregulation; ↑ upregulation; (+) positive expression; (-) negative expression.

Overall, the data available in the literature support the hypothesis of a major role of MCTs in the emergence of the hyper-glycolytic and acid-resistant phenotypes, as adaptations to the hypoxic microenvironment. The up-regulation of MCTs in the plasma membrane of different type of tumors is an adaptive mechanism to allow continuous high glycolytic rates, by exporting the accumulating end-product, lactate, as well as to counteract acid-induced apoptosis or necrosis. However, it was clear that this might not be the case for all tumor types, therefore, in most cases there are no functional studies showing the dependence of the tumors on MCT expression and activity. As so, additional studies on MCT expression in other tumor types, confirmation of the results already published as well as additional functional studies are needed to deeply understand the role of MCTs in cancer maintenance and aggressiveness and exactly in which cases these transporters could be used for therapy.

1.3.2 MCTs as suitable targets for cancer therapy. What is known?

Lately there was an increase in the exploitation of treatments that target tumour metabolism, being some of them already in clinical trial phase. Figure 8 shows actual and future therapeutic targets of tumour metabolism by targeting metabolic enzymes.

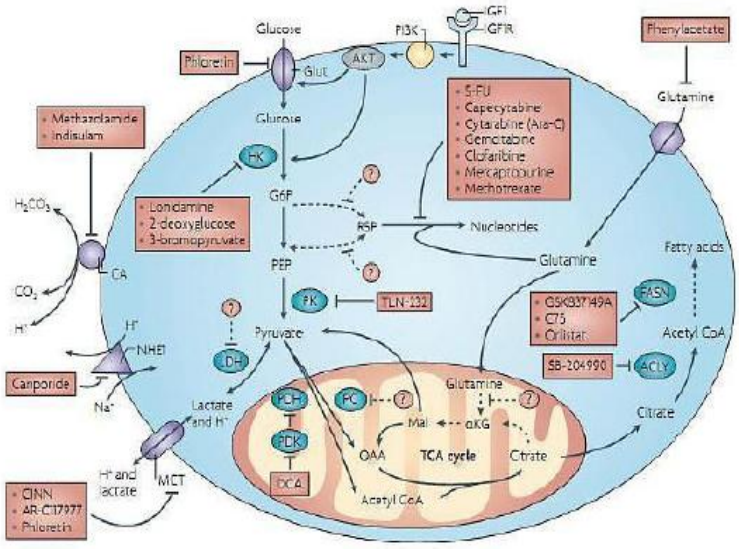

Figure 13. Actual and future therapeutic targets (dashed lines) of tumour metabolism by targeting metabolic enzymes [161].

Several studies have already shown the *in vitro* and *in vivo* effect of targeting different MCTs isoforms in human cancers.

It was demonstrated that MCT inhibition decreases intracellular pH [162], leads to cell death and, importantly, enhances cancer cell radiosensitivity [163]. Results for MCT4 show a decrease in cancer cell migration, by mechanisms that also involve interaction of MCT4 with β1-integrin [164]. In contrast, another study showed that silencing of MCT1 or MCT4 inhibited cancer cell invasion, but did not influence cell migration [165]. *In vivo* models have also been used, where administration of α-cyano-4-hydroxycinnamic acid (CHC), retarded tumor growth, rendered tumor cells sensitive to radiation, induced tumor necrosis and decreased tumor invasion [166]. The importance of MCTs for *in vivo* tumor growth was confirmed by a more specific approach, where combined silencing of MCT1 and MCT4 or silencing of CD147 significantly reduced glycolytic flux and tumor growth. There are also other MCT inhibitors described which are either non-isoform specific (ARC155858 targets both MCT1 and MCT2 [167] or target other molecules besides MCTs (e.g., lonidamine primary target is hexokinase II)) [167,168]. However, these compounds have been little explored as lactate transport inhibitors in the cancer context. In sum, it seems clear that MCTs represent a suitable target for cancer therapy; however, the effect of this inhibition seems highly dependent on the cell type and the isoform that is being targeted.

Figure 14 shows the model for therapeutic targeting of lactate-based metabolic symbiosis in tumours. It shows that MCTs inhibition would have a major effect on lactate transport, pH balance and tumour homeostasis, by compromising aerobic glycolysis and microenvironmental acidosis, as well as, the cell-cell lactate shuttle between aerobic and hypoxic cell populations.

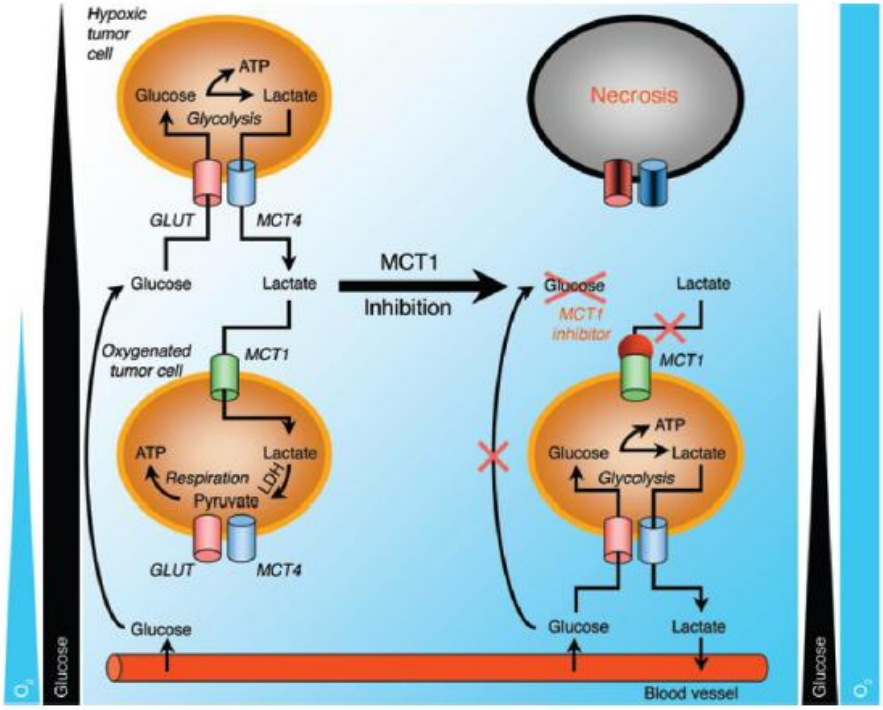

Figure 14. Model for therapeutic targeting of lactate-based metabolic symbiosis in tumours [118]

Regarding prostate cancer, which is our main focus in this thesis, few studies exist reporting the effect of MCTs inhibition in prostate cancer cells. As so, it is important to invest in studies evaluating both blocking and the silencing of MCTs in prostate cancer in order to reach a conclusion about the effectiveness of this therapeutic approach.

1.4 Prostate Cancer

1.4.1 Epidemiology of the disease

Prostate cancer is one of the most prevalent and incident cancers in the male population worldwide after lung cancer, and the fifth most common cancer overall. In Portugal, prostate cancer is the leading cancer among men with 5,140 estimated incident cases in 2008 [169, 170]. The patterns of incidence and mortality provide a number of interesting leads with peak incidences in the United States, Australia and the Scandinavian countries. Incidence rates

in Asian countries are generally low, but in recent years have risen proportionately more than in western countries. Incidence rates increased dramatically through the early 1990s [171].

In the late 1970s and early 1980s there was a rise in incidence in part, due to increased detection with more frequent surgical treatment for benign prostatic hyperplasia (BPH), particularly, transurethral resection of the prostate (TURP) [172]. Between 1986 and 1992 there was another sharp rise in incidence largely due to increasing use of prostate specific antigen (PSA) [173]. A recently published study points out an increase of 26% in the incidence of PCa in the period between 1986 to 2005 in the United States, which means that more than a million additional men were diagnosed with prostate cancer. Besides PSA screening programmes, other aspects such as the introduction of better diagnostic tools like transrectal ultrasonography (TRUS) –guided biopsy may be partially responsible for the increased incidence of prostate cancer in developed countries. However, screening practice differences alone are unlikely to explain the nearly huge differences in prostate cancer risk between high- and low- risk populations [174, 175].

Despite prostate cancer's high morbidity, its etiology remains obscure, with the only established risk factors being increasing age, race, and a family history. Many putative risk factors, including hormones, dietary factors, obesity, physical inactivity, occupation, vasectomy, smoking, sexual factors, and genetic susceptibility, have been implicated, but the epidemiologic evidence is inconclusive. While it is not known whether the risk factors explaining the observed patterns are environmental, lifestyle, or genetic, it is likely that a complex interplay of these factors is associated with prostate cancer development.

Concerning mortality, the rates are much less variable than PCa incidence, leading to a less accentuated difference between developed and developing countries. With the introduction of PSA a slight increase in mortality rates was observed but this increase was verified both in high and low risk countries [176]. The reasons for this time trends in mortality rates are still controversial but the introduction of the PSA screening was in certain way responsible for the

increasing rates due to miscertification of cause of death among men diagnosed with latent tumours, however it may also play a role in decreasing rates because of an earlier diagnosis of most of the cases.

Early detection in prostate cancer is crucial since only organ-confined disease is amenable to curative treatment whereas patients with advanced disease can only be palliated. Although there are some available tools frequently used for PCa detection, their performance is sub-optimal due to non-satisfactory sensitivity and specificity rates. In this context, new biomarkers are urgently needed, not only to early detection but also as ancillary tools for diagnosis, which is still based on histopathological evaluation of biopsy specimens.

1.4.2 Prostate Cancer Diagnosis

Usually, men with PSA levels of 4.0 ng/mL or greater are candidates to perform a prostatic core needle biopsy [177]. This cut off value for the PSA test is still controversial because PSA level screening produces false-negative or false-positive results that can lead men without disease to be submitted to unnecessary additional testing [177, 178]. Importantly, PSA levels screening are not acceptable diagnostic tools for PCa in routine clinical practice because they lack adequate sensitivity and specificity. Regardless the utility of diagnosis aid-tools, the only way to access diagnosis of prostate cancer is by histopathological analysis of prostatic tissue obtained from biopsy or prostatectomy specimens. It is crucial to perform a proper histopathological evaluation of the tissue collected from the patient in order to determine which are the best treatment options available for each patient because the combination of this evaluation results with other parameters, such as serum PSA levels, in nomograms do have prognostic impact [179]. Biopsy techniques have been improved in order to reduce morbidity and increase the rate and accuracy of detection in the first biopsy, although the optimal number of cores that must be collected is still a matter of debate [178].

1.4.2.1 Histophatological evaluation. The Gleason score system for prostate cancer grading

The Gleason grading system was firstly described in 1996 and it is presently, with some modifications, the most commonly used method for prostate cancer grading. Because the Gleason score (GS) is directly correlated with PCa prognosis, its accurate evaluation is critical to the natural history of the disease or the risk of recurrence following radical prostatectomy or radiotherapy [180].

The system is based on the evaluation of the glandular architectural patterns of the tumour tissue, recognizing five different grades that range from good to poor grade of differentiation. Because prostate adenocarcinomas are heterogeneous, more than one of the five patterns defined by Gleason might be present in the same tumour. To incorporate this heterogeneity in tumour grading, the Gleason score (GS) was developed, resulting from the sum of two most predominant cancer pattern (grades) in a sample. GS varies from 2 (1+1) to 10 (5+5) (Figure 15).

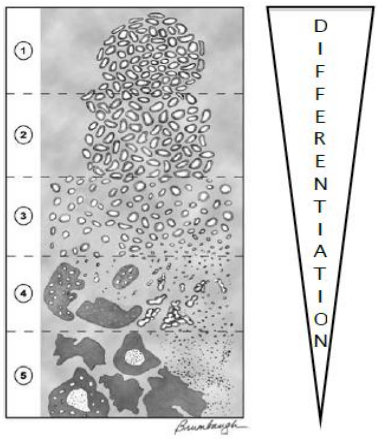

Figure 15. Updated Gleason score for histological grading of prostate tumours [181] .

Pattern 1- Closely packed small, uniform glands.
Pattern 2- More stroma between the glands that are round or oval with smooth ends and may minimally invade non-neoplastic tissue.
Pattern 3- Irregular size glands with angular shape and more infiltrative margins.
Pattern 4- Fused, cribiform or poorly defined glands.
Pattern 5- Only occasional gland formation.

1.4.2.2 Clinical and Pathological Staging

Another aspect that helps to predict the progression of PCa is the staging which comprises information about the extension of the disease (local, regional or systemic) and it is widely classified using the UICC (International Union against Cancer) Tumour Node Metastasis (TNM) system (Table 4). In this classification, T represents the extent of the primary tumour, N the lymph node status and M to

distant metastasis. The clinical stage is based on the evaluation of the patient by digital rectal exam (DRE), transrectal ultrasonography (TRUS), and, possibly, magnetic resonance imaging (MRI) [178,182,183] . Further information can be provided by biopsy histopathological evaluation and serum PSA levels [188]. Alternatively, pathological stage is determined after surgical removal of the prostate through adequate analysis of the prostatectomy specimen, and it predicts disease recurrence much more accurately [183]. There are several independent prognostic factors included in staging that are important to guide clinical decisions concerning the treatment options. These are extra-prostatic tumour invasion, seminal vesicles involvement, lymph node metastasis and distant metastasis. These features have been combined in nomograms with other prognostic factors, such as preoperative serum PSA levels, GS in the prostatectomy specimen and surgical margins *status* in an attempt to refine the prediction of prognosis

Table 4. Overview of the UICC TNM/pTNM staging system for prostate cancer [182] .

Primary Tumor (T)	
Tx	Primary Tumor cannot be assessed
T0	No evidence of primary tumor
T1	Clinically inapparent tumor neither palpable nor visible by imaging
T1a	Tumor incidental histologic finding in 5% or less of tissue resected
T1b	Tumor incidental histologic finding in more than 5% of tissue resected
T1c	Tumor identified by needle biopsy
T2	Tumor confined within prostate
T2a	Tumor involves one-half of one lobe or less
T2b	Tumor involves more than one-half of one lobe but not both lobes
T2c	Tumor involves both lobes
T3	Tumor extends through the prostate capsule
T3a	Extracapsular extension (unilateral or bilateral)
T3b	Tumor invades seminal vesicle(s)
T4	Tumor is fixed or invades adjacent structures other than seminal vesicles: such as external sphincter, rectum, bladder, levator muscles, and/or pelvic wall
Pathologic (pT) *	
pT2	Organ confined
pT2a	Unilateral, one-half of one side or less
pT3	Extraprostatic extension
pT3a	Extraprostatic extension or microscopic invasion of bladder neck
pT3b	Seminal vesicle invasion
pT4	Invasion of rectum, levator muscles and/or pelvic wall
Regional Lymph Nodes (N)	
Nx	Regional lymph nodes were not assessed
N0	No regional lymph node metastasis
N1	Metastasis in regional lymph node(s)
Pathologic (pN)	
pNx	Regional nodes not sampled
pN0	No positive regional nodes
pN1	Metastasis in regional node(s)
Distant Metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis
M1a	Non-regional lymph node(s)
M1b	Bone(s)
M1c	Other site(s) with or without bone disease

** There is no pathologic T1 classification.*

1.4.3 A brief overview in the treatment options for prostatic adenocarcinoma

TNM classification, GS, preoperative serum PSA levels, patients age, life expectancy and quality of life are parameters that must be taken into account previous to therapeutic decision in order to classify the patient according to risk of disease progression and mortality [184]. After taking into account the evaluation of these features several therapeutic options are available for PCa patients, namely watchful waiting (active surveillance), surgery, radiotherapy, and hormone therapy [179].

Watchful waiting is a viable option for some prostate cancers because the natural history of this disease is often long and many patients diagnosed with early stage PCa are more prone to die with, rather than from, the disease [185]. So, for men with less than 10 years of life expectancy and with low-risk tumours (i.e., tumours highly to moderately differentiated – GS < 7, stage T1c to T2a and serum PSA equal to or less than 10ng/mL) active surveillance should be offered as well as for patients with relevant comorbidities or with asymptomatic metastatic disease and a strong wish to avoid treatment-related side effects, it is also an option [184].

Radical prostatectomy is the standard curative treatment for prostate cancer. Of all the treatments available, it has a survival advantage when compared to active surveillance. The improvement of surgical techniques resulted in high cure rates with reduced morbidities (e.g. erectile dysfunction or urinary incontinence), which are frequent in patients submitted to radical prostatectomy [179].

Hormone therapy is used in men with advanced prostate cancer to shrink the cancer and slow the growth of tumors. In men with early-stage prostate cancer, hormone therapy may be used to shrink tumors before radiation therapy. This can make it more likely that radiation therapy will be successful. Hormone therapy is sometimes used after surgery or radiation therapy to slow the growth of any cancer cells left behind. Blocking the supply of hormones may cause cancer cells to die or to grow more slowly. Hormone therapy options include medications that stop your body from producing testosterone as luteinizing hormone-releasing hormone (LH-RH) agonists prevent the testicles from receiving messages to make testosterone. Drugs typically used in this type of hormone therapy include leuprolide (Lupron, Eligard), goserelin (Zoladex), triptorelin (Trelstar) and histrelin

(Vantas), medications that block testosterone from reaching cancer cells. Medications known as anti-androgens prevent testosterone from reaching cancer cells. Examples include bicalutamide (Casodex), flutamide, and nilutamide (Nilandron). These drugs typically are given along with an LH-RH agonist or given before taking an LH-RH agonist. Also surgery to remove the testicles (orchiectomy) reduces testosterone levels in the body. Orchiectomy in lowering testosterone levels is similar to that of hormone therapy medications, but orchiectomy may lower testosterone levels more quickly.

However, most tumours submitted to androgen deprivation acquire a hormone-refractory phenotype. For these patients the only available options are conventional chemotherapy, whose effectiveness is still limited [178, 184]. More recently, hormonal therapy has been evaluated as adjuvant therapy in combination with radical prostatectomy or radiotherapy, but only for the latter case it shows an improvement in survival [179].

Several major clinical challenges are associated with this conventional paradigm for prostate cancer diagnosis and treatment, as so, the major aim is still the search for new prospects to answer old challenges.

1.4.4 What is known about prostate cancer metabolism?

As it was mentioned above most malignancies have increased glycolysis for energy requirement of rapid cell proliferation, which is the basis for tumor imaging through glucose analog 2-deoxy-2-fluoro-D-glucose (FDG) with positron emission tomography. Indeed there are studies reporting the presence of hypoxia in PCa, pointing to the presence of a glycolytic phenotype in prostate cancer cells [186-189]. In contrast, other authors have shown that one of the significant characteristics of prostate cancer is slow glycolysis and low FDG activity. Also, another study revealed that GLUT1 mRNA and protein were only very weakly expressed in human prostate cancer tissue, which is considered to account for low FDG activity of prostate cancer [190]. As so, FDG-PET has not been very helpful in staging or detection of recurrence of prostate cancer because of low glycolysis.

Clinical trials showed that FDG accumulation does not even correlate with increasing grade or stage of the tumor and there is a significant overlap in FDG uptake between benign prostate hyperplasia and prostate cancer [191, 192]. Other positron-emitting radiotracers, such as ^{18}F-choline, ^{11}C-choline, ^{11}C-acetate and ^{18}F-fluorodihydrotestosterone have recently been suggested as putative imaging agents for the detection of prostate tumors [191-195]. Clinical evaluation of these radiotracers is presently underway and the role of PET scanning in prostate cancer is still evolving [190,196,197].

Studies suggest that increased citrate oxidation is a significant metabolic characteristic for the bioenergy requirement in prostate cancer. The normal human prostate gland, has the function of producing, accumulating and secrete high levels of citrate [198, 199] and in contrast to normal glands and benign hyperplasia, prostate cancer is characterized by a low levels of citrate (Figure 16). Based on bioenergetics of prostate epithelial cell metabolism, Costello and Franklin proposed the "bioenergetic theory of prostate malignancy": the transformation of a citrate-producing sane epithelial cell to a malignant citrate-oxidating cell that would result in a more efficient energy-generating system. Additionally, the authors suggest that in order to meet the energetic requirements of malignant cells, the metabolic transformation into citrate oxidation must be an early event in preparation for the progression of malignancy and preceded the histopathologic identification of malignant cells [198, 200, 201] . In fact, changes of citrate levels in the form of choline/citrate for detection and localization of prostate cancer is a basis of magnetic resonance spectroscopy (MRS) for *in situ* detection of prostate cancer [202, 203]. Recent studies showed that activity of mitochondrial (m)-aconitase, the first reaction before citrate oxidation, is significantly higher in prostate cancer compared to normal prostate, which drives the utilization of citrate as energy source [198].

In order to maintain this net citrate production, continuous availability of oxaloacetate and acetyl-CoA is required for continuous citrate synthesis. Acetyl-coenzyme A is the only molecule consumed in citrate cycle and its continuous availability is crucial for driving citrate oxidation. Recent studies also suggested that to meet the bioenergetic requirement for rapid cell proliferation in prostate

cancer there are changes of fatty acid metabolism that provide both ATP and acetyl-CoA to make the acceleration of citrate oxidation possible.

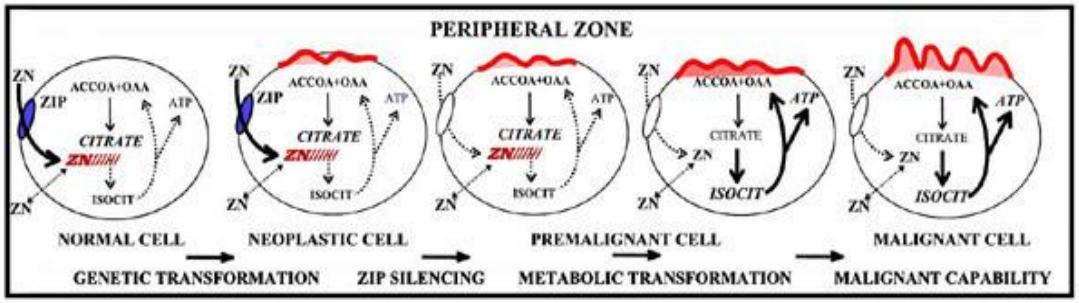

Figure 16. Concept of pathogenesis of prostate malignancy. The high zinc levels in mitochondria inhibit m-aconitase resulting in the inability to oxidize citrate and the accumulation of citrate. As the cellular zink levels are decreased in the premalignant cell, citrate oxidation occurs with the concurrent increased production of ATP. The malignant cell is now metabolically and energetically capable of proceeding with its malignant process (From [202]).

Although some evidence supports a crucial role of fatty acid-related metabolism in the pathogenesis and progression of prostate malignancy [204, 205], the reports are scarce with low number of clinical samples and with no information regarding the clinico-pathological significance of these alterations; as so, PCa metabolism is still largely unknown and consequently which metabolic pathway represents the most appropriate target for metabolic inhibition in PCa is still controversial.

1.5 REFERENCES

1. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**(1): p. 57-70.
2. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.
3. Warburg, O., F. Wind, and E. Negelein, *The Metabolism of Tumors in the Body.* J Gen Physiol, 1927. **8**(6): p. 519-30.
4. Vander Heiden, M.G., L.C. Cantley, and C.B. Thompson, *Understanding the Warburg effect: the metabolic requirements of cell proliferation.* Science, 2009. **324**(5930): p. 1029-33.
5. Jones, R.G. and C.B. Thompson, *Tumor suppressors and cell metabolism: a recipe for cancer growth.* Genes Dev, 2009. **23**(5): p. 537-48.
6. DeBerardinis, R.J., et al., *The biology of cancer: metabolic reprogramming fuels cell growth and proliferation.* Cell Metab, 2008. **7**(1): p. 11-20.
7. Hsu, P.P. and D.M. Sabatini, *Cancer cell metabolism: Warburg and beyond.* Cell, 2008. **134**(5): p. 703-7.
8. Phelps, M.E., *PET: the merging of biology and imaging into molecular imaging.* J Nucl Med, 2000. **41**(4): p. 661-81.
9. Ganapathy, V., M. Thangaraju, and P.D. Prasad, *Nutrient transporters in cancer: relevance to Warburg hypothesis and beyond.* Pharmacol Ther, 2009. **121**(1): p. 29-40.
10. Kroemer, G. and J. Pouyssegur, *Tumor cell metabolism: cancer's Achilles' heel.* Cancer Cell, 2008. **13**(6): p. 472-82.
11. Gatenby, R.A. and R.J. Gillies, *Why do cancers have high aerobic glycolysis?* Nat Rev Cancer, 2004. **4**(11): p. 891-9.
12. Moreno-Sanchez, R., et al., *Energy metabolism in tumor cells.* FEBS J, 2007. **274**(6): p. 1393-418.
13. Gillies, R.J. and R.A. Gatenby, *Adaptive landscapes and emergent phenotypes: why do cancers have high glycolysis?* J Bioenerg Biomembr, 2007. **39**(3): p. 251-7.
14. Pouyssegur, J., F. Dayan, and N.M. Mazure, *Hypoxia signalling in cancer and approaches to enforce tumour regression.* Nature, 2006. **441**(7092): p. 437-43.
15. Koukourakis, M.I., et al., *Comparison of metabolic pathways between cancer cells and stromal cells in colorectal carcinomas: a metabolic survival role for tumor-associated stroma.* Cancer Res, 2006. **66**(2): p. 632-7.
16. Swietach, P., R.D. Vaughan-Jones, and A.L. Harris, *Regulation of tumor pH and the role of carbonic anhydrase 9.* Cancer Metastasis Rev, 2007. **26**(2): p. 299-310.
17. Fischer, K., et al., *Inhibitory effect of tumor cell-derived lactic acid on human T cells.* Blood, 2007. **109**(9): p. 3812-9.
18. Brandon, M., P. Baldi, and D.C. Wallace, *Mitochondrial mutations in cancer.* Oncogene, 2006. **25**(34): p. 4647-62.
19. Wallace, D.C., *Mitochondria and cancer: Warburg addressed.* Cold Spring Harb Symp Quant Biol, 2005. **70**: p. 363-74.
20. Semenza, G.L., *Hypoxia-inducible factor 1 and the molecular physiology of oxygen homeostasis.* J Lab Clin Med, 1998. **131**(3): p. 207-14.

21. Semenza, G.L., *Hypoxia-inducible factor 1: master regulator of O2 homeostasis.* Curr Opin Genet Dev, 1998. **8**(5): p. 588-94.
22. Semenza, G.L., *Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1.* Annu Rev Cell Dev Biol, 1999. **15**: p. 551-78.
23. Semenza, G.L., *Hypoxia, clonal selection, and the role of HIF-1 in tumor progression.* Crit Rev Biochem Mol Biol, 2000. **35**(2): p. 71-103.
24. Semenza, G.L., *Hypoxia-inducible factor 1: control of oxygen homeostasis in health and disease.* Pediatr Res, 2001. **49**(5): p. 614-7.
25. Greijer, A.E., et al., *Up-regulation of gene expression by hypoxia is mediated predominantly by hypoxia-inducible factor 1 (HIF-1).* J Pathol, 2005. **206**(3): p. 291-304.
26. Cassavaugh, J. and K.M. Lounsbury, *Hypoxia-mediated biological control.* J Cell Biochem, 2011. **112**(3): p. 735-44.
27. Chiche, J., M.C. Brahimi-Horn, and J. Pouyssegur, *Tumour hypoxia induces a metabolic shift causing acidosis: a common feature in cancer.* J Cell Mol Med, 2010. **14**(4): p. 771-94.
28. Gillies, R.J., I. Robey, and R.A. Gatenby, *Causes and consequences of increased glucose metabolism of cancers.* J Nucl Med, 2008. **49 Suppl 2**: p. 24S-42S.
29. Harris, A.L., *Hypoxia--a key regulatory factor in tumour growth.* Nat Rev Cancer, 2002. **2**(1): p. 38-47.
30. Pastorekova, S., P.J. Ratcliffe, and J. Pastorek, *Molecular mechanisms of carbonic anhydrase IX-mediated pH regulation under hypoxia.* BJU Int, 2008. **101 Suppl 4**: p. 8-15.
31. Porporato, P.E., et al., *Anticancer targets in the glycolytic metabolism of tumors: a comprehensive review.* Front Pharmacol, 2011. **2**: p. 49.
32. Quennet, V., et al., *Tumor lactate content predicts for response to fractionated irradiation of human squamous cell carcinomas in nude mice.* Radiother Oncol, 2006. **81**(2): p. 130-5.
33. Brizel, D.M., et al., *Elevated tumor lactate concentrations predict for an increased risk of metastases in head-and-neck cancer.* Int J Radiat Oncol Biol Phys, 2001. **51**(2): p. 349-53.
34. Paschen, W., et al., *Lactate and pH in the brain: association and dissociation in different pathophysiological states.* J Neurochem, 1987. **48**(1): p. 154-9.
35. Walenta, S., T. Schroeder, and W. Mueller-Klieser, *Metabolic mapping with bioluminescence: basic and clinical relevance.* Biomol Eng, 2002. **18**(6): p. 249-62.
36. Kumar, V.B., et al., *Endothelial cell response to lactate: implication of PAR modification of VEGF.* J Cell Physiol, 2007. **211**(2): p. 477-85.
37. Stern, R., et al., *Lactate stimulates fibroblast expression of hyaluronan and CD44: the Warburg effect revisited.* Exp Cell Res, 2002. **276**(1): p. 24-31.
38. Rudrabhatla, S.R., C.L. Mahaffey, and M.E. Mummert, *Tumor microenvironment modulates hyaluronan expression: the lactate effect.* J Invest Dermatol, 2006. **126**(6): p. 1378-87.
39. Baumann, F., et al., *Lactate promotes glioma migration by TGF-beta2-dependent regulation of matrix metalloproteinase-2.* Neuro Oncol, 2009. **11**(4): p. 368-80.
40. Mazurek, S., et al., *Tumor M2-PK and glutaminolytic enzymes in the metabolic shift of tumor cells.* Anticancer Res, 2000. **20**(6D): p. 5151-4.

41. DeBerardinis, R.J., et al., *Beyond aerobic glycolysis: transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis.* Proc Natl Acad Sci U S A, 2007. **104**(49): p. 19345-50.
42. Juel, C. and A.P. Halestrap, *Lactate transport in skeletal muscle - role and regulation of the monocarboxylate transporter.* J Physiol, 1999. **517 (Pt 3)**: p. 633-42.
43. Halestrap, A.P. and R.M. Denton, *Specific inhibition of pyruvate transport in rat liver mitochondria and human erythrocytes by alpha-cyano-4-hydroxycinnamate.* Biochem J, 1974. **138**(2): p. 313-6.
44. Juel, C. and A.P. Halestrap, *Lactate transport in skeletal muscle - role and regulation of the monocarboxylate transporter.* J Physiol, 1999. **517 (Pt 3)**: p. 633-42.
45. Halestrap, A.P. and N.T. Price, *The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation.* Biochem J, 1999. **343 Pt 2**: p. 281-99.
46. Juel, C., *Lactate-proton cotransport in skeletal muscle.* Physiol Rev, 1997. **77**(2): p. 321-58.
47. Halestrap, A.P. and D. Meredith, *The SLC16 gene family-from monocarboxylate transporters (MCTs) to aromatic amino acid transporters and beyond.* Pflugers Arch, 2004. **447**(5): p. 619-28.
48. Price, N.T., V.N. Jackson, and A.P. Halestrap, *Cloning and sequencing of four new mammalian monocarboxylate transporter (MCT) homologues confirms the existence of a transporter family with an ancient past.* Biochem J, 1998. **329 (Pt 2)**: p. 321-8.
49. Carpenter, L., R.C. Poole, and A.P. Halestrap, *Cloning and sequencing of the monocarboxylate transporter from mouse Ehrlich Lettre tumour cell confirms its identity as MCT1 and demonstrates that glycosylation is not required for MCT1 function.* Biochim Biophys Acta, 1996. **1279**(2): p. 157-63.
50. Yoon, H., et al., *Identification of a unique monocarboxylate transporter (MCT3) in retinal pigment epithelium.* Biochem Biophys Res Commun, 1997. **234**(1): p. 90-4.
51. Yoon, H. and N.J. Philp, *Genomic structure and developmental expression of the chicken nonocarboxylate transporter MCT3 gene.* Exp Eye Res, 1998. **67**(4): p. 417-24.
52. de Bruijne, A.W., H. Vreeburg, and J. van Steveninck, *Alternative-substrate inhibition of L-lactate transport via the monocarboxylate-specific carrier system in human erythrocytes.* Biochim Biophys Acta, 1985. **812**(3): p. 841-4.
53. Lin, R.Y., et al., *Human monocarboxylate transporter 2 (MCT2) is a high affinity pyruvate transporter.* J Biol Chem, 1998. **273**(44): p. 28959-65.
54. Dimmer, K.S., et al., *The low-affinity monocarboxylate transporter MCT4 is adapted to the export of lactate in highly glycolytic cells.* Biochem J, 2000. **350 Pt 1**: p. 219-27.
55. De Bruijne, A.W., H. Vreeburg, and J. Van Steveninck, *Kinetic analysis of L-lactate transport in human erythrocytes via the monocarboxylate-specific carrier system.* Biochim Biophys Acta, 1983. **732**(3): p. 562-8.

56. Philp, N.J., H. Yoon, and E.F. Grollman, *Monocarboxylate transporter MCT1 is located in the apical membrane and MCT3 in the basal membrane of rat RPE.* Am J Physiol, 1998. **274**(6 Pt 2): p. R1824-8.
57. Pinheiro, C., et al., *Role of monocarboxylate transporters in human cancers: state of the art.* J Bioenerg Biomembr, 2012. **44**(1): p. 127-39.
58. Philp, N.J., et al., *Loss of MCT1, MCT3, and MCT4 expression in the retinal pigment epithelium and neural retina of the 5A11/basigin-null mouse.* Invest Ophthalmol Vis Sci, 2003. **44**(3): p. 1305-11.
59. Cuff, M.A. and S.P. Shirazi-Beechey, *The human monocarboxylate transporter, MCT1: genomic organization and promoter analysis.* Biochem Biophys Res Commun, 2002. **292**(4): p. 1048-56.
60. Fishbein, W.N., N. Merezhinskaya, and J.W. Foellmer, *Relative distribution of three major lactate transporters in frozen human tissues and their localization in unfixed skeletal muscle.* Muscle Nerve, 2002. **26**(1): p. 101-12.
61. Hashimoto, T., R. Hussien, and G.A. Brooks, *Colocalization of MCT1, CD147, and LDH in mitochondrial inner membrane of L6 muscle cells: evidence of a mitochondrial lactate oxidation complex.* Am J Physiol Endocrinol Metab, 2006. **290**(6): p. E1237-44.
62. Yoshida, Y., et al., *Negligible direct lactate oxidation in subsarcolemmal and intermyofibrillar mitochondria obtained from red and white rat skeletal muscle.* J Physiol, 2007. **582**(Pt 3): p. 1317-35.
63. McClelland, G.B., et al., *Peroxisomal membrane monocarboxylate transporters: evidence for a redox shuttle system?* Biochem Biophys Res Commun, 2003. **304**(1): p. 130-5.
64. Jackson, V.N., et al., *Cloning of the monocarboxylate transporter isoform MCT2 from rat testis provides evidence that expression in tissues is species-specific and may involve post-transcriptional regulation.* Biochem J, 1997. **324 (Pt 2)**: p. 447-53.
65. Garcia, C.K., et al., *cDNA cloning of MCT2, a second monocarboxylate transporter expressed in different cells than MCT1.* J Biol Chem, 1995. **270**(4): p. 1843-9.
66. Gerhart, D.Z., R.L. Leino, and L.R. Drewes, *Distribution of monocarboxylate transporters MCT1 and MCT2 in rat retina.* Neuroscience, 1999. **92**(1): p. 367-75.
67. Pierre, K., et al., *Cell-specific localization of monocarboxylate transporters, MCT1 and MCT2, in the adult mouse brain revealed by double immunohistochemical labeling and confocal microscopy.* Neuroscience, 2000. **100**(3): p. 617-27.
68. Pierre, K., P.J. Magistretti, and L. Pellerin, *MCT2 is a major neuronal monocarboxylate transporter in the adult mouse brain.* J Cereb Blood Flow Metab, 2002. **22**(5): p. 586-95.
69. Broer, S., et al., *Characterization of the high-affinity monocarboxylate transporter MCT2 in Xenopus laevis oocytes.* Biochem J, 1999. **341 (Pt 3)**: p. 529-35.
70. Debernardi, R., et al., *Cell-specific expression pattern of monocarboxylate transporters in astrocytes and neurons observed in different mouse brain cortical cell cultures.* J Neurosci Res, 2003. **73**(2): p. 141-55.

71. Pellerin, L., et al., *Cellular and subcellular distribution of monocarboxylate transporters in cultured brain cells and in the adult brain.* J Neurosci Res, 2005. **79**(1-2): p. 55-64.
72. Benton, C.R., et al., *Monocarboxylate transporters in subsarcolemmal and intermyofibrillar mitochondria.* Biochem Biophys Res Commun, 2004. **323**(1): p. 249-53.
73. Manning Fox, J.E., D. Meredith, and A.P. Halestrap, *Characterisation of human monocarboxylate transporter 4 substantiates its role in lactic acid efflux from skeletal muscle.* J Physiol, 2000. **529 Pt 2**: p. 285-93.
74. Friesema, E.C., et al., *Identification of monocarboxylate transporter 8 as a specific thyroid hormone transporter.* J Biol Chem, 2003. **278**(41): p. 40128-35.
75. Friesema, E.C., et al., *Effective cellular uptake and efflux of thyroid hormone by human monocarboxylate transporter 10.* Mol Endocrinol, 2008. **22**(6): p. 1357-69.
76. Friesema, E.C., et al., *Association between mutations in a thyroid hormone transporter and severe X-linked psychomotor retardation.* Lancet, 2004. **364**(9443): p. 1435-7.
77. Dumitrescu, A.M., et al., *A novel syndrome combining thyroid and neurological abnormalities is associated with mutations in a monocarboxylate transporter gene.* Am J Hum Genet, 2004. **74**(1): p. 168-75.
78. Brockmann, K., et al., *X-linked paroxysmal dyskinesia and severe global retardation caused by defective MCT8 gene.* J Neurol, 2005. **252**(6): p. 663-6.
79. Boccone, L., et al., *Allan-Herndon-Dudley syndrome (AHDS) in two consecutive generations caused by a missense MCT8 gene mutation. Phenotypic variability with the presence of normal serum T3 levels.* Eur J Med Genet, 2013. **56**(4): p. 207-10.
80. Maranduba, C.M., et al., *Decreased cellular uptake and metabolism in Allan-Herndon-Dudley syndrome (AHDS) due to a novel mutation in the MCT8 thyroid hormone transporter.* J Med Genet, 2006. **43**(5): p. 457-60.
81. Murakami, Y., et al., *Functional characterization of human monocarboxylate transporter 6 (SLC16A5).* Drug Metab Dispos, 2005. **33**(12): p. 1845-51.
82. Kolz, M., et al., *Meta-analysis of 28,141 individuals identifies common variants within five new loci that influence uric acid concentrations.* PLoS Genet, 2009. **5**(6): p. e1000504.
83. Chung, W., et al., *Identification of novel tumor markers in prostate, colon and breast cancer by unbiased methylation profiling.* PLoS One, 2008. **3**(4): p. e2079.
84. Morris, M.E. and M.A. Felmlee, *Overview of the proton-coupled MCT (SLC16A) family of transporters: characterization, function and role in the transport of the drug of abuse gamma-hydroxybutyric acid.* AAPS J, 2008. **10**(2): p. 311-21.
85. Bonen, A., et al., *Abundance and subcellular distribution of MCT1 and MCT4 in heart and fast-twitch skeletal muscles.* Am J Physiol Endocrinol Metab, 2000. **278**(6): p. E1067-77.
86. Coles, L., et al., *Exercise rapidly increases expression of the monocarboxylate transporters MCT1 and MCT4 in rat muscle.* J Physiol, 2004. **561**(Pt 1): p. 253-61.

87. Cuff, M.A., D.W. Lambert, and S.P. Shirazi-Beechey, *Substrate-induced regulation of the human colonic monocarboxylate transporter, MCT1.* J Physiol, 2002. **539**(Pt 2): p. 361-71.
88. Hashimoto, T., et al., *Lactate sensitive transcription factor network in L6 cells: activation of MCT1 and mitochondrial biogenesis.* FASEB J, 2007. **21**(10): p. 2602-12.
89. Kang, K.W., M.J. Jin, and H.K. Han, *IGF-I receptor gene activation enhanced the expression of monocarboxylic acid transporter 1 in hepatocarcinoma cells.* Biochem Biophys Res Commun, 2006. **342**(4): p. 1352-5.
90. Baker, S.K., K.J. McCullagh, and A. Bonen, *Training intensity-dependent and tissue-specific increases in lactate uptake and MCT-1 in heart and muscle.* J Appl Physiol, 1998. **84**(3): p. 987-94.
91. Pilegaard, H., et al., *Effect of high-intensity exercise training on lactate/H+ transport capacity in human skeletal muscle.* Am J Physiol, 1999. **276**(2 Pt 1): p. E255-61.
92. Dubouchaud, H., et al., *Endurance training, expression, and physiology of LDH, MCT1, and MCT4 in human skeletal muscle.* Am J Physiol Endocrinol Metab, 2000. **278**(4): p. E571-9.
93. Green, H., et al., *Increases in muscle MCT are associated with reductions in muscle lactate after a single exercise session in humans.* Am J Physiol Endocrinol Metab, 2002. **282**(1): p. E154-60.
94. Juel, C., et al., *Effect of high-intensity intermittent training on lactate and H+ release from human skeletal muscle.* Am J Physiol Endocrinol Metab, 2004. **286**(2): p. E245-51.
95. Thomas, C., et al., *Monocarboxylate transporters, blood lactate removal after supramaximal exercise, and fatigue indexes in humans.* J Appl Physiol, 2005. **98**(3): p. 804-9.
96. Burgomaster, K.A., et al., *Divergent response of metabolite transport proteins in human skeletal muscle after sprint interval training and detraining.* Am J Physiol Regul Integr Comp Physiol, 2007. **292**(5): p. R1970-6.
97. Mohr, M., et al., *Effect of two different intense training regimens on skeletal muscle ion transport proteins and fatigue development.* Am J Physiol Regul Integr Comp Physiol, 2007. **292**(4): p. R1594-602.
98. Thibault, R., et al., *Down-regulation of the monocarboxylate transporter 1 is involved in butyrate deficiency during intestinal inflammation.* Gastroenterology, 2007. **133**(6): p. 1916-27.
99. Rafiki, A., et al., *Highly differential expression of the monocarboxylate transporters MCT2 and MCT4 in the developing rat brain.* Neuroscience, 2003. **122**(3): p. 677-88.
100. Leino, R.L., D.Z. Gerhart, and L.R. Drewes, *Monocarboxylate transporter (MCT1) abundance in brains of suckling and adult rats: a quantitative electron microscopic immunogold study.* Brain Res Dev Brain Res, 1999. **113**(1-2): p. 47-54.
101. Hatta, H., et al., *Tissue-specific and isoform-specific changes in MCT1 and MCT4 in heart and soleus muscle during a 1-yr period.* Am J Physiol Endocrinol Metab, 2001. **281**(4): p. E749-56.

102. Okamura, H., S.S. Spicer, and B.A. Schulte, *Developmental expression of monocarboxylate transporter in the gerbil inner ear.* Neuroscience, 2001. **107**(3): p. 499-505.
103. Baud, O., et al., *Perinatal and early postnatal changes in the expression of monocarboxylate transporters MCT1 and MCT2 in the rat forebrain.* J Comp Neurol, 2003. **465**(3): p. 445-54.
104. Leino, R.L., et al., *Diet-induced ketosis increases monocarboxylate transporter (MCT1) levels in rat brain.* Neurochem Int, 2001. **38**(6): p. 519-27.
105. Hadjiagapiou, C., et al., *Role of USF1 and USF2 as potential repressor proteins for human intestinal monocarboxylate transporter 1 promoter.* Am J Physiol Gastrointest Liver Physiol, 2005. **288**(6): p. G1118-26.
106. Borthakur, A., et al., *Regulation of monocarboxylate transporter 1 (MCT1) promoter by butyrate in human intestinal epithelial cells: involvement of NF-kappaB pathway.* J Cell Biochem, 2008. **103**(5): p. 1452-63.
107. Saksena, S., et al., *PKC-dependent stimulation of the human MCT1 promoter involves transcription factor AP2.* Am J Physiol Gastrointest Liver Physiol, 2009. **296**(2): p. G275-83.
108. Benton, C.R., et al., *PGC-1alpha increases skeletal muscle lactate uptake by increasing the expression of MCT1 but not MCT2 or MCT4.* Physiol Genomics, 2008. **35**(1): p. 45-54.
109. Konig, B., et al., *Monocarboxylate transporter (MCT)-1 is up-regulated by PPARalpha.* Biochim Biophys Acta, 2008. **1780**(6): p. 899-904.
110. Konig, B., et al., *Monocarboxylate transporter 1 and CD147 are up-regulated by natural and synthetic peroxisome proliferator-activated receptor alpha agonists in livers of rodents and pigs.* Mol Nutr Food Res, 2010. **54**(9): p. 1248-56.
111. Coller, H.A., et al., *Expression analysis with oligonucleotide microarrays reveals that MYC regulates genes involved in growth, cell cycle, signaling, and adhesion.* Proc Natl Acad Sci U S A, 2000. **97**(7): p. 3260-5.
112. Fang, J., et al., *The H+-linked monocarboxylate transporter (MCT1/SLC16A1): a potential therapeutic target for high-risk neuroblastoma.* Mol Pharmacol, 2006. **70**(6): p. 2108-15.
113. Kang, K.W., et al., *C-myc amplification altered the gene expression of ABC- and SLC-transporters in human breast epithelial cells.* Mol Pharm, 2009. **6**(2): p. 627-33.
114. McClelland, G.B. and G.A. Brooks, *Changes in MCT 1, MCT 4, and LDH expression are tissue specific in rats after long-term hypobaric hypoxia.* J Appl Physiol, 2002. **92**(4): p. 1573-84.
115. Zhang, F., et al., *Monocarboxylate transporter expression in the spontaneous hypertensive rat: effect of stroke.* J Neurosci Res, 2005. **79**(1-2): p. 139-45.
116. Ord, J.J., et al., *Comparison of hypoxia transcriptome in vitro with in vivo gene expression in human bladder cancer.* Br J Cancer, 2005. **93**(3): p. 346-54.
117. Kay, H.H., S. Zhu, and S. Tsoi, *Hypoxia and lactate production in trophoblast cells.* Placenta, 2007. **28**(8-9): p. 854-60.
118. Sonveaux, P., et al., *Targeting lactate-fueled respiration selectively kills hypoxic tumor cells in mice.* J Clin Invest, 2008. **118**(12): p. 3930-42.

119. Perez de Heredia, F., I.S. Wood, and P. Trayhurn, *Hypoxia stimulates lactate release and modulates monocarboxylate transporter (MCT1, MCT2, and MCT4) expression in human adipocytes.* Pflugers Arch, 2010. **459**(3): p. 509-18.
120. Proud, C.G., *Regulation of mRNA translation.* Essays Biochem, 2001. **37**: p. 97-108.
121. Li, K.K., et al., *miR-124 is frequently down-regulated in medulloblastoma and is a negative regulator of SLC16A1.* Hum Pathol, 2009. **40**(9): p. 1234-43.
122. Wilson, M.C., et al., *Basigin (CD147) is the target for organomercurial inhibition of monocarboxylate transporter isoforms 1 and 4: the ancillary protein for the insensitive MCT2 is EMBIGIN (gp70).* J Biol Chem, 2005. **280**(29): p. 27213-21.
123. Deora, A.A., et al., *Mechanisms regulating tissue-specific polarity of monocarboxylate transporters and their chaperone CD147 in kidney and retinal epithelia.* Proc Natl Acad Sci U S A, 2005. **102**(45): p. 16245-50.
124. Biswas, C., et al., *The human tumor cell-derived collagenase stimulatory factor (renamed EMMPRIN) is a member of the immunoglobulin superfamily.* Cancer Res, 1995. **55**(2): p. 434-9.
125. Wilson, M.C., D. Meredith, and A.P. Halestrap, *Fluorescence resonance energy transfer studies on the interaction between the lactate transporter MCT1 and CD147 provide information on the topology and stoichiometry of the complex in situ.* J Biol Chem, 2002. **277**(5): p. 3666-72.
126. Slomiany, M.G., et al., *Hyaluronan, CD44, and emmprin regulate lactate efflux and membrane localization of monocarboxylate transporters in human breast carcinoma cells.* Cancer Res, 2009. **69**(4): p. 1293-301.
127. Buyse, M., et al., *Luminal leptin enhances CD147/MCT-1-mediated uptake of butyrate in the human intestinal cell line Caco2-BBE.* J Biol Chem, 2002. **277**(31): p. 28182-90.
128. Pierre, K., et al., *Noradrenaline enhances monocarboxylate transporter 2 expression in cultured mouse cortical neurons via a translational regulation.* J Neurochem, 2003. **86**(6): p. 1468-76.
129. Wang, Y., et al., *T3 increases lactate transport and the expression of MCT4, but not MCT1, in rat skeletal muscle.* Am J Physiol Endocrinol Metab, 2003. **285**(3): p. E622-8.
130. Fanelli, A., et al., *MCT1 and its accessory protein CD147 are differentially regulated by TSH in rat thyroid cells.* Am J Physiol Endocrinol Metab, 2003. **285**(6): p. E1223-9.
131. Saksena, S., et al., *Mechanisms underlying modulation of monocarboxylate transporter 1 (MCT1) by somatostatin in human intestinal epithelial cells.* Am J Physiol Gastrointest Liver Physiol, 2009. **297**(5): p. G878-85.
132. Murray, C.M., et al., *Monocarboxylate transporter MCT1 is a target for immunosuppression.* Nat Chem Biol, 2005. **1**(7): p. 371-6.
133. Guile, S.D., et al., *Potent blockers of the monocarboxylate transporter MCT1: novel immunomodulatory compounds.* Bioorg Med Chem Lett, 2006. **16**(8): p. 2260-5.
134. Davis, C.D. and J.A. Milner, *Biomarkers for diet and cancer prevention research: potentials and challenges.* Acta Pharmacol Sin, 2007. **28**(9): p. 1262-73.

135. Naderi, G.A., et al., *Anti-oxidant effect of flavonoids on the susceptibility of LDL oxidation.* Mol Cell Biochem, 2003. **246**(1-2): p. 193-6.
136. Mamani-Matsuda, M., et al., *Therapeutic and preventive properties of quercetin in experimental arthritis correlate with decreased macrophage inflammatory mediators.* Biochem Pharmacol, 2006. **72**(10): p. 1304-10.
137. Lotito, S.B. and B. Frei, *Dietary flavonoids attenuate tumor necrosis factor alpha-induced adhesion molecule expression in human aortic endothelial cells. Structure-function relationships and activity after first pass metabolism.* J Biol Chem, 2006. **281**(48): p. 37102-10.
138. Piantelli, M., et al., *Flavonoids inhibit melanoma lung metastasis by impairing tumor cells endothelium interactions.* J Cell Physiol, 2006. **207**(1): p. 23-9.
139. Vijayababu, M.R., et al., *Quercetin downregulates matrix metalloproteinases 2 and 9 proteins expression in prostate cancer cells (PC-3).* Mol Cell Biochem, 2006. **287**(1-2): p. 109-16.
140. Tan, W.F., et al., *Quercetin, a dietary-derived flavonoid, possesses antiangiogenic potential.* Eur J Pharmacol, 2003. **459**(2-3): p. 255-62.
141. Cunningham, P., I. Afzal-Ahmed, and R.J. Naftalin, *Docking studies show that D-glucose and quercetin slide through the transporter GLUT1.* J Biol Chem, 2006. **281**(9): p. 5797-803.
142. Strobel, P., et al., *Myricetin, quercetin and catechin-gallate inhibit glucose uptake in isolated rat adipocytes.* Biochem J, 2005. **386**(Pt 3): p. 471-8.
143. Park, J.B. and M. Levine, *Intracellular accumulation of ascorbic acid is inhibited by flavonoids via blocking of dehydroascorbic acid and ascorbic acid uptakes in HL-60, U937 and Jurkat cells.* J Nutr, 2000. **130**(5): p. 1297-302.
144. Belt, J.A., et al., *Inhibition of lactate transport and glycolysis in Ehrlich ascites tumor cells by bioflavonoids.* Biochemistry, 1979. **18**(16): p. 3506-11.
145. Deuticke, B., *Monocarboxylate transport in red blood cells: kinetics and chemical modification.* Methods Enzymol, 1989. **173**: p. 300-29.
146. Wang, Q. and M.E. Morris, *Flavonoids modulate monocarboxylate transporter-1-mediated transport of gamma-hydroxybutyrate in vitro and in vivo.* Drug Metab Dispos, 2007. **35**(2): p. 201-8.
147. Floridi, A., et al., *Lonidamine, a selective inhibitor of aerobic glycolysis of murine tumor cells.* J Natl Cancer Inst, 1981. **66**(3): p. 497-9.
148. Pulselli, R., et al., *Effect of lonidamine on the mitochondrial potential in situ in Ehrlich ascites tumor cells.* Anticancer Res, 1996. **16**(1): p. 419-23.
149. Ben-Horin, H., et al., *Mechanism of action of the antineoplastic drug lonidamine: 31P and 13C nuclear magnetic resonance studies.* Cancer Res, 1995. **55**(13): p. 2814-21.
150. Ben-Yoseph, O., et al., *Mechanism of action of lonidamine in the 9L brain tumor model involves inhibition of lactate efflux and intracellular acidification.* J Neurooncol, 1998. **36**(2): p. 149-57.
151. Di Cosimo, S., et al., *Lonidamine: efficacy and safety in clinical trials for the treatment of solid tumors.* Drugs Today (Barc), 2003. **39**(3): p. 157-74.
152. Pinheiro, C., et al., *Increased expression of monocarboxylate transporters 1, 2, and 4 in colorectal carcinomas.* Virchows Arch, 2008. **452**(2): p. 139-46.

153. Pinheiro, C., et al., *Increasing expression of monocarboxylate transporters 1 and 4 along progression to invasive cervical carcinoma.* Int J Gynecol Pathol, 2008. **27**(4): p. 568-74.
154. Pinheiro, C., et al., *Monocarboxylate transporter 1 is up-regulated in basal-like breast carcinoma.* Histopathology, 2010. **56**(7): p. 860-7.
155. Pinheiro, C., et al., *Expression of monocarboxylate transporters 1, 2, and 4 in human tumours and their association with CD147 and CD44.* J Biomed Biotechnol, 2010. **2010**: p. 427694.
156. Koukourakis, M.I., et al., *Lung cancer: a comparative study of metabolism related protein expression in cancer cells and tumor associated stroma.* Cancer Biol Ther, 2007. **6**(9): p. 1476-9.
157. Ritzhaupt, A., et al., *Identification of a monocarboxylate transporter isoform type 1 (MCT1) on the luminal membrane of human and pig colon.* Biochem Soc Trans, 1998. **26**(2): p. S120.
158. Lambert, D.W., et al., *Molecular changes in the expression of human colonic nutrient transporters during the transition from normality to malignancy.* Br J Cancer, 2002. **86**(8): p. 1262-9.
159. Chen, H., et al., *Co-expression of CD147/EMMPRIN with monocarboxylate transporters and multiple drug resistance proteins is associated with epithelial ovarian cancer progression.* Clin Exp Metastasis, 2010. **27**(8): p. 557-69.
160. Pinheiro, C., et al., *The prognostic value of CD147/EMMPRIN is associated with monocarboxylate transporter 1 co-expression in gastric cancer.* Eur J Cancer, 2009. **45**(13): p. 2418-24.
161. Tennant, D.A., R.V. Duran, and E. Gottlieb, *Targeting metabolic transformation for cancer therapy.* Nat Rev Cancer, 2010. **10**(4): p. 267-77.
162. Wahl, M.L., et al., *Regulation of intracellular pH in human melanoma: potential therapeutic implications.* Mol Cancer Ther, 2002. **1**(8): p. 617-28.
163. Colen, C.B., et al., *Metabolic remodeling of malignant gliomas for enhanced sensitization during radiotherapy: an in vitro study.* Neurosurgery, 2006. **59**(6): p. 1313-23; discussion 1323-4.
164. Gallagher, S.M., J.J. Castorino, and N.J. Philp, *Interaction of monocarboxylate transporter 4 with beta1-integrin and its role in cell migration.* Am J Physiol Cell Physiol, 2009. **296**(3): p. C414-21.
165. Izumi, H., et al., *Monocarboxylate transporters 1 and 4 are involved in the invasion activity of human lung cancer cells.* Cancer Sci, 2011. **102**(5): p. 1007-13.
166. Colen, C.B., et al., *Metabolic targeting of lactate efflux by malignant glioma inhibits invasiveness and induces necrosis: an in vivo study.* Neoplasia, 2011. **13**(7): p. 620-32.
167. Ovens, M.J., et al., *AR-C155858 is a potent inhibitor of monocarboxylate transporters MCT1 and MCT2 that binds to an intracellular site involving transmembrane helices 7-10.* Biochem J, 2010. **425**(3): p. 523-30.
168. Le Floch, R., et al., *CD147 subunit of lactate/H+ symporters MCT1 and hypoxia-inducible MCT4 is critical for energetics and growth of glycolytic tumors.* Proc Natl Acad Sci U S A, 2011. **108**(40): p. 16663-8.
169. Center, M.M., et al., *International variation in prostate cancer incidence and mortality rates.* Eur Urol, 2012. **61**(6): p. 1079-92.
170. Heidenreich, A., et al., *EAU guidelines on prostate cancer.* Eur Urol, 2008. **53**(1): p. 68-80.

171. Hsing, A.W., L. Tsao, and S.S. Devesa, *International trends and patterns of prostate cancer incidence and mortality.* Int J Cancer, 2000. **85**(1): p. 60-7.
172. Potosky, A.L., et al., *Rise in prostatic cancer incidence associated with increased use of transurethral resection.* J Natl Cancer Inst, 1990. **82**(20): p. 1624-8.
173. Potosky, A.L., et al., *The role of increasing detection in the rising incidence of prostate cancer.* JAMA, 1995. **273**(7): p. 548-52.
174. Boyle, P., P. Maisonneuve, and P. Napalkov, *Geographical and temporal patterns of incidence and mortality from prostate cancer.* Urology, 1995. **46**(3 Suppl A): p. 47-55.
175. Quinn, M. and P. Babb, *Patterns and trends in prostate cancer incidence, survival, prevalence and mortality. Part II: individual countries.* BJU Int, 2002. **90**(2): p. 174-84.
176. Quinn, M. and P. Babb, *Patterns and trends in prostate cancer incidence, survival, prevalence and mortality. Part I: international comparisons.* BJU Int, 2002. **90**(2): p. 162-73.
177. Wolf, A.M., et al., *American Cancer Society guideline for the early detection of prostate cancer: update 2010.* CA Cancer J Clin, 2010. **60**(2): p. 70-98.
178. Heidenreich, A., et al., *[EAU guidelines on prostate cancer. Part I: screening, diagnosis, and treatment of clinically localised disease].* Actas Urol Esp, 2011. **35**(9): p. 501-14.
179. Jani, A.B. and S. Hellman, *Early prostate cancer: clinical decision-making.* Lancet, 2003. **361**(9362): p. 1045-53.
180. Epstein, J.I., *Update on the Gleason grading system.* Ann Pathol, 2011. **31**(5 Suppl): p. S20-6.
181. Epstein, J.I., *An update of the Gleason grading system.* J Urol, 2010. **183**(2): p. 433-40.
182. Edge, S.B. and C.C. Compton, *The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM.* Ann Surg Oncol, 2010. **17**(6): p. 1471-4.
183. Hoedemaeker, R.F., A.N. Vis, and T.H. Van Der Kwast, *Staging prostate cancer.* Microsc Res Tech, 2000. **51**(5): p. 423-9.
184. Damber, J.E. and G. Aus, *Prostate cancer.* Lancet, 2008. **371**(9625): p. 1710-21.
185. Frankel, S., et al., *Screening for prostate cancer.* Lancet, 2003. **361**(9363): p. 1122-8.
186. Higgins, L.H., et al., *Hypoxia and the metabolic phenotype of prostate cancer cells.* Biochim Biophys Acta, 2009. **1787**(12): p. 1433-43.
187. Effert, P., et al., *Expression of glucose transporter 1 (Glut-1) in cell lines and clinical specimens from human prostate adenocarcinoma.* Anticancer Res, 2004. **24**(5A): p. 3057-63.
188. Stewart, G.D., et al., *Analysis of hypoxia-associated gene expression in prostate cancer: lysyl oxidase and glucose transporter-1 expression correlate with Gleason score.* Oncol Rep, 2008. **20**(6): p. 1561-7.
189. Vaz, C.V., et al., *Androgen-responsive and nonresponsive prostate cancer cells present a distinct glycolytic metabolism profile.* Int J Biochem Cell Biol, 2012. **44**(11): p. 2077-84.
190. Nanni, C., et al., *11C/ 18F-choline PET or 11C/18F-acetate PET in prostate cancer: may a choice be recommended?* Eur J Nucl Med Mol Imaging, 2007. **34**(10): p. 1704-5.

191. Vavere, A.L., et al., *1-11C-acetate as a PET radiopharmaceutical for imaging fatty acid synthase expression in prostate cancer.* J Nucl Med, 2008. **49**(2): p. 327-34.
192. Garg, S., et al., *In vivo biodistribution of an androgen receptor avid PET imaging agent 7-alpha-fluoro-17 alpha-methyl-5-alpha-dihydrotestosterone ([(18)F]FMDHT) in rats pretreated with cetrorelix, a GnRH antagonist.* Eur J Nucl Med Mol Imaging, 2008. **35**(2): p. 379-85.
193. Schilling, D., et al., *Histological verification of 11C-choline-positron emission/computed tomography-positive lymph nodes in patients with biochemical failure after treatment for localized prostate cancer.* BJU Int, 2008. **102**(4): p. 446-51.
194. Reske, S.N., *[11C]Choline uptake with PET/CT for the initial diagnosis of prostate cancer: relation to PSA levels, tumour stage and anti-androgenic therapy.* Eur J Nucl Med Mol Imaging, 2008. **35**(9): p. 1740-1.
195. Soloviev, D., et al., *PET imaging with 11C-acetate in prostate cancer: a biochemical, radiochemical and clinical perspective.* Eur J Nucl Med Mol Imaging, 2008. **35**(5): p. 942-9.
196. Takahashi, N., et al., *The roles of PET and PET/CT in the diagnosis and management of prostate cancer.* Oncology, 2007. **72**(3-4): p. 226-33.
197. Bouchelouche, K. and P. Oehr, *Recent developments in urologic oncology: positron emission tomography molecular imaging.* Curr Opin Oncol, 2008. **20**(3): p. 321-6.
198. Costello, L.C., et al., *Zinc inhibition of mitochondrial aconitase and its importance in citrate metabolism of prostate epithelial cells.* J Biol Chem, 1997. **272**(46): p. 28875-81.
199. Liu, Y., L.C. Costello, and R.B. Franklin, *Prolactin specifically regulates citrate oxidation and m-aconitase of rat prostate epithelial cells.* Metabolism, 1996. **45**(4): p. 442-9.
200. Cooper, J.F. and I. Farid, *The Role of Citric Acid in the Physiology of the Prostate. 3. Lactate/Citrate Ratios in Benign and Malignant Prostatic Homogenates as an Index of Prostatic Malignancy.* J Urol, 1964. **92**: p. 533-6.
201. Costello, L.C. and R.B. Franklin, *The intermediary metabolism of the prostate: a key to understanding the pathogenesis and progression of prostate malignancy.* Oncology, 2000. **59**(4): p. 269-82.
202. Costello, L.C., et al., *Role of zinc in the pathogenesis and treatment of prostate cancer: critical issues to resolve.* Prostate Cancer Prostatic Dis, 2004. **7**(2): p. 111-7.
203. Kurhanewicz, J., et al., *Three-dimensional H-1 MR spectroscopic imaging of the in situ human prostate with high (0.24-0.7-cm3) spatial resolution.* Radiology, 1996. **198**(3): p. 795-805.
204. Zha, S., et al., *Peroxisomal branched chain fatty acid beta-oxidation pathway is upregulated in prostate cancer.* Prostate, 2005. **63**(4): p. 316-23.
205. Liu, Y., L.S. Zuckier, and N.V. Ghesani, *Dominant uptake of fatty acid over glucose by prostate cells: a potential new diagnostic and therapeutic approach.* Anticancer Res, 2010. **30**(2): p. 369-74.

CHAPTER 2. DIAGNOSTIC/PROGNOSTIC VALUE OF MCTs AND CHAPERONES EXPRESSION IN PROSTATE CANCER

2.1. CHAPTER OVERVIEW

Most malignancies rely on glycolysis for rapid proliferation even in the presence of oxygen, leading to production of large amounts of acids, mainly lactic acid. Consequently, the high glycolytic phenotype induces an acidic tumour environment, which is associated with the increase of several malignant features including cellular migration, invasion and metastization.

The evidence for the upregulation of MCTs in several solid tumours, such as colorectal carcinomas, uterine cervix carcinomas, melanomas, breast carcinomas and lung tumours was already described, however, in prostate carcinoma, the role of MCTs was unknown.

In this chapter, MCT1, MCT2, MCT4, Gp70 and CD147 expressions were evaluated in a comprehensive series including 171 patients, who performed radical prostatectomy and 14 patients who performed cystoprostatectomy. Samples and clinico-pathological data were retrieved and organized into tissue microarray (TMAs) blocks. Protein expression was evaluated by immunohistochemistry in neoplastic (n = 171), adjacent non-neoplastic tissues (n = 135), PIN lesions (n = 40) and normal prostatic tissue (n = 14). Protein expression was correlated with patients' clinicopathologic characteristics.
In this study, already published in an international scientific periodical with referees, a significant increase of MCT2 and MCT4 expressions was observed. Importantly, while MCT1 was observed at the plasma membrane, MCT2 and MCT4 were in the cytoplasm. Also, analysis of MCT expression in regard to the clinico-pathological parameters showed associations of MCT4 and CD147 expression and reliable markers of poor prognosis such as PSA levels, Gleason score and pT stage, as well as with perineural invasion and biochemical recurrence. Additionally, we also verified an involvement of MCTs 1 and 4 with proteins associated with drug resistance and cancer progression, which could imply that MCTs are involved in these two processes.

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2.2 PUBLISHED RESULTS

The results presented in this chapter were:

(i) Published as an original article in an international peer reviewed journal

Pértega-Gomes N, Vizcaíno JR, Miranda-Gonçalves V, Pinheiro C, Silva J, Pereira H, Monteiro P, Henrique RM, Reis RM, Lopes C, Baltazar F. "Monocarboxylate transporter 4 (MCT4) and CD147 overexpression is associated with poor prognosis in prostate cancer." **BMC Cancer** 2011 Jul 25;11:312. (HIGHLY ACCESSED) (Citations ISI: 19).

(ii) Selected for publication as an abstract in international scientific journals

Pértega-Gomes N, Vizcaíno JR, Lopes C, Baltazar F. "Monocarboxylate transporters as targets for cancer therapy". Abstract **published** in conference proceedings **Microsc Microanal** 2009;15: 17-18, Supplement S3.

Pértega-Gomes N, Vizcaíno JR, Lopes C, Baltazar F. "Monocarboxylate transporters 2 and 4 are upregulated in prostate carcinoma". Abstract **published** in conference proceedings **Virchows Arch** 2009,455:233, Supplement: Suppl. 1.

(iii) Presented as oral communication in the following national scientific meeting:

Pértega-Gomes N, Vizcaíno JR, Lopes C, Baltazar F. "Characterization of Monocarboxylate transporters in prostate cancer". XIII Workshop of Urological Oncology, 2010, Espinho, Portugal.

(iv) Presented as <u>poster in the following international scientific meetings</u>:

Pértega-Gomes N, Vizcaíno JR, Lopes C, Baltazar F. et al. "Assessment of monocarboxylate transporters 1 and 4, CD147, CD44 and MDRI could help to predict biochemical recurrence in prostate cancer". XX Porto Cancer Meeting, IPATIMUP, Porto, Portugal, 2011.

(v) Were <u>recognized with the following prizes/awards:</u>

Pértega-Gomes N, Vizcaíno JR, Lopes C and Baltazar F. "Characterization of Monocarboxylate transporters in prostate cancer". **Honorable Mention** for **best scientific poster** and **oral communication** on the XIII Workshop of Urological Oncology, 2010, Espinho, Portugal.

2.2.1 Monocarboxylate transporter 4 (MCT4) and CD147 overexpression is associated with poor prognosis in Prostate Cancer

BMC Cancer

RESEARCH ARTICLE Open Access

Monocarboxylate transporter 4 (MCT4) and CD147 overexpression is associated with poor prognosis in prostate cancer

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Abstract

Background: Monocarboxylate transporters (MCTs) are transmembrane proteins involved in the transport of monocarboxylates across the plasma membrane, which appear to play an important role in solid tumours, however the role of MCTs in prostate cancer is largely unknown. The aim of the present work was to evaluate the clinico-pathological value of monocarboxylate transporters (MCTs) expression, namely MCT1, MCT2 and MCT4, together with CD147 and gp70 as MCT1/4 and MCT2 chaperones, respectively, in prostate carcinoma.

Methods: Prostate tissues were obtained from 171 patients, who performed radical prostatectomy and 14 patients who performed cystoprostatectomy. Samples and clinico-pathological data were retrieved and organized into tissue microarray (TMAs) blocks. Protein expression was evaluated by immunohistochemistry in neoplastic (n = 171), adjacent non-neoplastic tissues (n = 135), PIN lesions (n = 40) and normal prostatic tissue (n = 14). Protein expression was correlated with patients' clinicopathologic characteristics.

Results: In the present study, a significant increase of MCT2 and MCT4 expression in the cytoplasm of tumour cells and a significant decrease in both MCT1 and CD147 expression in prostate tumour cells was observed when compared to normal tissue. All MCT isoforms and CD147 were expressed in PIN lesions. Importantly, for MCT2 and MCT4 the expression levels in PIN lesions were between normal and tumour tissue, which might indicate a role for these MCTs in the malignant transformation. Associations were found between MCT1, MCT4 and CD147 expressions and poor prognosis markers; importantly MCT4 and CD147 overexpression correlated with higher PSA levels, Gleason score and pT stage, as well as with perineural invasion and biochemical recurrence.

Conclusions: Our data provides novel evidence for the involvement of MCTs in prostate cancer. According to our results, we consider that MCT2 should be further explored as tumour marker and both MCT4 and CD147 as markers of poor prognosis in prostate cancer.

Background

Increased glucose consumption is a hallmark of malignant cells, which is responsible for energy production from glycolysis [1]. Most malignancies rely on this pathway for rapid proliferation even in the presence of oxygen, leading to production of large amounts of acids, mainly lactic acid [1,2]. Consequently, the high glycolytic phenotype induces an acidic tumour environment, which is associated with the increase of several malignant features including cellular migration, invasion and metastisation [2].

In order to prevent cell death by cellular acidosis, tumour cells increase proton efflux through pH regulators such as proton-pumps, sodium-proton exchangers, bicarbonate transporters and monocarboxylate transporters (MCTs) [3]. MCTs are proteins that facilitate the transmembrane transport of short-chain fatty acids, such as pyruvate and lactate, coupled with a proton. In glycolytic tumours, they promote the efflux of lactic acid, constituting important players in the maintenance

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of tumour intracellular pH, as well as in the maintenance of the high rates of glycolysis [4,5]. Therefore, MCTs play a central role in tumour metabolism and, as a result, constitute attractive targets in cancer therapy which have not been explored yet.

We and others have shown evidence for the upregulation of MCTs in several solid tumours, such as colorectal carcinomas [6], uterine cervix carcinomas [7], melanomas [8], breast carcinomas [9,10] and lung tumours [11]. However, in prostate carcinoma, the role of MCTs is largely unknown. To the best of our knowledge, we were the first to report MCT expression in prostate cancer [12,13], however, there is a recent study [14] evaluating the role of MCTs in prostate drug resistance and progression but this study does not evaluate neither MCT isoform 2 (MCT2) nor gp70, its known chaperone.

MCT expression appears to be influenced by altered physiologic conditions, however the underlying molecular events involved in MCT regulation are still poorly understood. Recently, it was demonstrated that proper expression and activity of MCT1 and MCT4 requires co-expression of CD147, also known as EMMPRIN or basigin [15-17]. On the other hand, *in vitro* studies showed that maturation and cell surface expression of CD147 is also dependent on MCT1 and MCT4 expressions [18,19]. MCT2 expression and activity depends on a different chaperone known as gp70.

CD147 alone has already been described as a key element in oncogenesis by stimulating the synthesis of several matrix metalloproteinases, leading to enhanced tumour cell invasion [20,21]. This protein is described to be up-regulated in tumours, including prostate cancer, where it has been identified as an unfavourable prognosis marker [22-25]. However, the role of CD147/MCT co-expression in prostate cancer is far from understood.

The aim of the present study was to assess the role of MCTs in prostate cancer, by comparing the immunohistochemical expression of the MCT isoforms 1, 2 and 4, along with CD147 and gp70, in normal prostatic tissue, adjacent non-neoplastic tissue, PIN lesions and neoplastic tissues in a large series of prostate samples organized into tissue microarrays (TMAs), and evaluating their clinico-pathological value.

Methods

Case selection and TMA construction

Prostate tissues were obtained from 171 patients with a median age of 64 years old (range 46-74), who performed radical prostatectomy between 1993 and 2003. Samples and clinico-pathological data were retrieved from the files of the Department of Pathology, Centro Hospitalar do Porto and Centro Hospitalar do Alto Ave-Guimarães, and organized into 13 tissue microarray blocks (TMAs).

Prior to TMA construction, hematoxylin and eosin (H&E) tumour sections of each specimen of radical prostatectomy were re-assessed using the 2005 modified Gleason and 2010 p TNM AJCC classification [26,27]. Representative areas of adjacent non-neoplastic prostate tissue, PIN lesions and prostate cancer were selected. Adjacent non-neoplastic tissue samples and PIN lesions were selected from the peripheral zone of prostate were cancer develops. Each case was represented in the TMA by three cores (1 mm diameter) with 0.8 mm from core centre to core centre, and precisely deposited into a recipient paraffin block, using a TMA workstation (TMA builder, Beecher Instruments Inc. Technology). 4 μm tissue sections were used for immunohistochemistry (IHC) and H&E-stained section from each TMA block was reviewed to confirm the presence of morphological representative areas of the original tissues.

Normal prostate tissue was obtained from cystoprostatectomy cases and immunohistochemical expression was performed in the entire section of the fragments.

MCT and CD147 immunohistochemistry

IHC for MCTs was performed according to avidin-biotin-peroxidase complex principle (R.T.U. Vectastain Elite ABC Kit [Universal], Vector Laboratories, Burlingame, CA), with the primary antibodies for MCT1 (AB3538P, Chemicon International, Temecula, CA), MCT2 (sc-14926, Santa Cruz Biotechnology, Santa Cruz, CA) and MCT4 (AB3316P, Chemicon International, Temecula, CA), diluted 1:200, as previously described [6,7,9,10].

CD147 and gp70 IHC was performed according to the same principle (Ultravision Detection System Antipolyvalent, horseradish peroxidase; Lab Vision Corporation), with the primary antibody diluted 1:750, as previously described [9,10] for CD147 (18-7344, ZYMED Laboratories Inc., South San Francisco, CA) and diluted 1:100 for gp70 (HPA017740, Atlas Antibodies).

Negative controls were performed with appropriate serum controls for the primary antibodies (X0907 and N1699, Dako, Carpinteria, CA). Normal colon, kidney and skeletal muscle tissue were used as positive controls for MCT1, MCT2 and MCT4, respectively, cervical squamous carcinoma for CD147 and seminal vesicle for gp70. Tissue sections were counterstained with hematoxylin.

Immunohistochemical evaluation

Immunoreaction in TMA sections was evaluated for cytoplasmic and/or plasma membrane staining. Shortly, sections were scored semi-quantitatively as follows: 0: 0% of immunoreactive cells; 1: < 5% of immunoreactive cells; 2: 5-50% of immunoreactive cells and 3: > 50% of immunoreactive cells. Also, intensity of staining was

scored semi-quantitatively as follows: 0: negative; 1: weak; 2: moderate and 3: strong. The final score was defined as the sum of both parameters (extension and intensity), and grouped as negative (scores 0-3) and positive (scores 4-6). Discordant results in different cores of the same case were scored as follows: average of extension plus highest intensity score. Immunohistochemical evaluation was performed by two independent and experienced pathologists (JRV, PM), who were blind to the clinico-pathological data of the patients. Discordant results were discussed in a double-head microscope. Since staining was different among the positive cases, to further clarify the significance of the immunoexpression of MCTs and CD147 in prostate carcinoma, we categorized the cases in two groups: intermediate score group (ISG, score 4) and high score group (HSG, scores 5-6).

Statistics

Statistical analysis was performed using the SPSS statistical software (version 17.0, SPSS Inc., Chicago, IL, USA). All comparisons were examined for statistical significance using Pearson's chisquare (χ^2) test, being the threshold for significance $p < 0.05$.

Ethics

The work has been approved by DEFI (Departamento de Ensino Formação e Investigação) Ethics Committee of Centro Hospitalar do Porto ref. no. 017/08(010-DEFI/015-CES).

Results

MCT, CD147 and gp70 expressions in prostate tissues

A total of 346 prostate samples organised into TMAs (tissue microarrays), including 135 non-neoplastic, 40 PIN lesions and 171 neoplastic tissues were analysed for MCT1, MCT2, MCT4, CD147 and gp70 expressions. Also, 14 normal prostate tissues were analysed for MCTs, CD147 and gp70 expressions. We used a combined scoring system, previously described [6,7,9,10]. To better illustrate the scoring system used, representative images of positive cases with intensity score 1 (weak), score 2 (moderate) and score 3 (strong), for MCT1, MCT2, MCT4 and CD147 staining are shown in Figure 1.

Figure 2 summarises MCT and CD147 expressions in normal, adjacent non-neoplastic, PIN lesions and tumour tissues. A significant increase in both MCT2 and MCT4 expressions was observed from non-neoplastic (normal or adjacent) to tumour tissues ($p < 0.001$, for both) while a decrease was observed for MCT1 expression in the transition from normal or adjacent non-neoplastic to prostate tumour tissue ($p = 0.003$ and $p < 0.001$, respectively). CD147 expression decreased from normal to tumour tissue ($p = 0.006$), however, no significant differences were observed when compared to adjacent non-neoplastic tissue ($p = 0.236$). For MCT1 expression, we observed a decrease from PIN lesions to malignant glands ($p < 0.001$) with no significant differences between normal or adjacent non-neoplastic tissue and PIN lesions ($p = 0.545$ and $p = 0.063$, respectively). For MCT2, there was an increase from both normal and adjacent non-neoplastic tissue to PIN lesions ($p < 0.001$ and $p = 0.005$, respectively) whereas no significant differences were observed between PIN lesions and tumour ($p = 0.605$). There was a significant increase in MCT4 expression from normal to PIN lesions ($p = 0.024$) and from PIN lesions to tumour ($p = 0.022$) but not between adjacent non-neoplastic tissue and PIN lesions ($p = 0.410$). For CD147, there was a significant decrease from normal tissue to PIN lesions ($p = 0.043$) but no difference between adjacent non-neoplastic tissue and PIN lesions ($p = 0.389$). No differences were observed between CD147 expression in PIN lesions and tumour ($p = 0.180$). Gp70 was negative in all normal, adjacent non-neoplastic and PIN lesions and only a very small percentage of cases (n = 4) were positive in tumours (data not shown).

Figure 3 shows representative immunohistochemical reactions for MCT1, MCT2, MCT4 and CD147 in normal, PIN lesions and tumour tissue. Staining for MCT1 and CD147 was mainly observed in the basal and lateral epithelial cell membranes, with negative immunoreactions in the apical zone of both normal glands (Figure 3A, J) PIN lesions (Figure 3B, K) and neoplastic cells (Figure 3C, L). MCT2 and MCT4 staining was cytoplasmic, with granular appearance, which was more evident for MCT2 (Figure 1F). Due to the epithelial nature of prostate carcinoma, positive immunoreactions were only considered for staining in epithelial cells.

As stated in Materials and Methods section, we stratified the positive cases into two groups, ISG (intermediate score group) and HSG (high score group). Although the number of normal prostate tissue cases is small, the differences between normal and tumour cases was evident, however, this difference was not so clear between adjacent non-neoplastic tissue and tumour. Thus, we compared the expression of the proteins in the ISG and HSG (Table 1). For both MCT1 and MCT4, there was a significant difference between neoplastic and adjacent non-neoplastic tissues only for the HSG. For MCT2, HSG predominated in neoplastic cells, whereas ISG was more frequent in adjacent non-neoplastic tissue. There were no differences in the distribution of CD147 final score between neoplastic and adjacent non neoplastic tissues.

We further assessed the association between CD147 and MCT expressions in the prostate cancer tissues (Table 2). This analysis showed that CD147 correlated with both MCT1 and MCT4 ($p < 0.001$ for both), but not with MCT2. Figure 3 (C, I and L) shows staining for

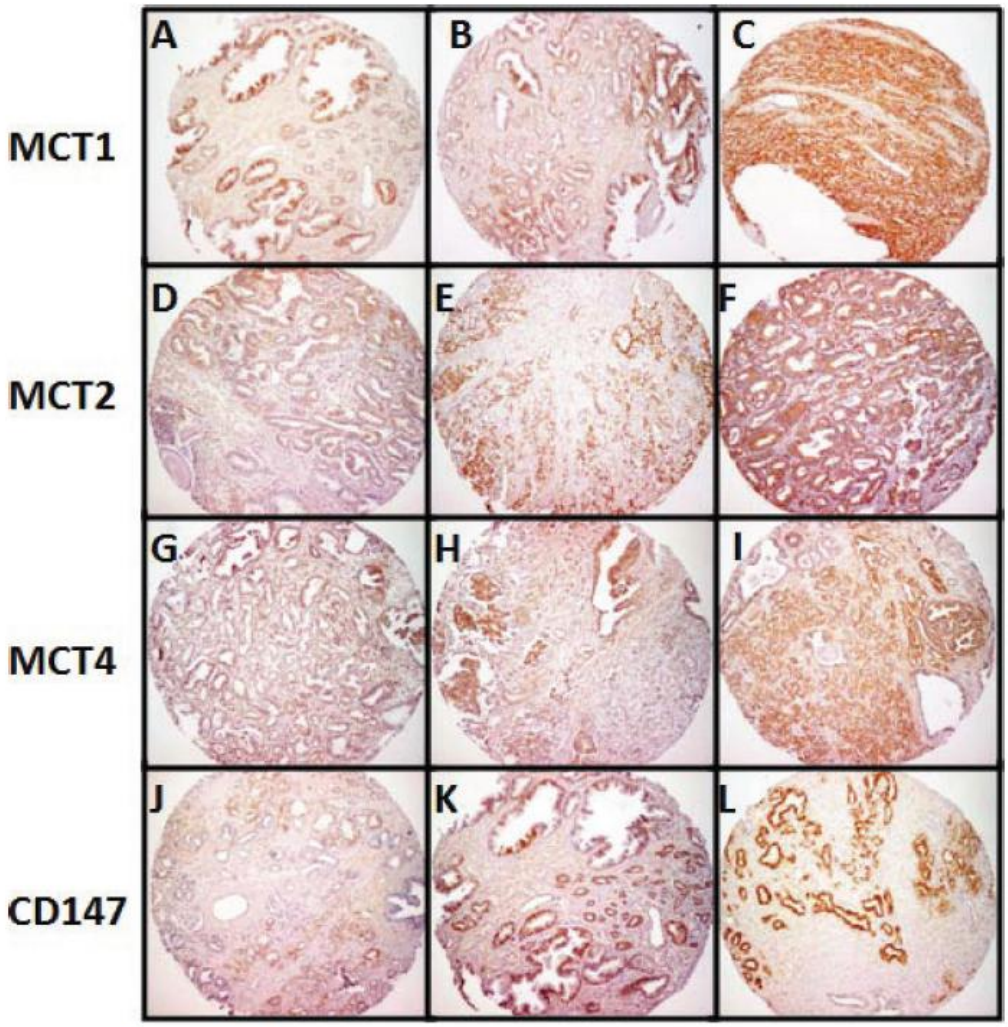

Figure 1 Expression of MCT1, MCT2, MCT4 and CD147 in prostate cancer tissue microarrays. Representative images of intensity score 1 (weak) for MCT1 (A), MCT2 (D), MCT4 (G) and CD147 (J), intensity score 2 (moderate) for MCT1 (B), MCT2 (E), MCT4 (H) and CD147 (K) and intensity score 3 (strong) for MCT1 (C), MCT2 (F), MCT4 (I) and CD147 (L) immunostaining for positive cases of prostate carcinoma (score ≥ 4).

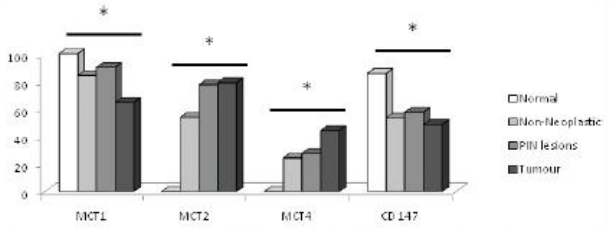

Figure 2 Frequency of MCTs and CD147 expressions in normal prostate, non-neoplastic, PIN lesions and tumour samples. In general, there is an increase in both MCT2 and MCT4 expressions from non-neoplastic (normal or adjacent) to tumour tissues, while a decrease is observed for MCT1 and CD147 expression in the transition from non-neoplastic (normal or adjacent) to prostate tumour tissue. See text for detail. * $p < 0.05$ (non-neoplastic adjacent, PIN and tumour tissue compared to normal tissue).

MCT1, MCT4 and CD147 in the same tumour area of one prostate tumour case, in which positive cells for the three proteins can be seen. No associations between gp70 and MCTs were found (data not shown).

Associations between MCTs and CD147 expressions and the clinic-pathological data

Assessment of association between MCTs and CD147 expressions and the clinico-pathological data is presented in Table 3. We found positive associations between MCT1 expression in the HSG and higher PSA levels ($p = 0.016$), absence of perineural invasion ($p = 0.036$) and presence of biochemical recurrence ($p = 0.047$). For MCT2, there was only an association with lower age at diagnosis for ISG ($p = 0.023$). MCT4 expression in the HSG was associated with higher age

Table 1 Distribution of positive final immunohistochemical score of monocarboxylate transporters (MCTs) and CD147 in adjacent non-tumoural (NT) and tumour tissue (T) of prostate samples

		n	ISG (%) (Score 4)	p	HSG (%) (Scores 5-6)	p
MCT1				0.500		**0.002**
	NT	**121**	17.4		66.9	
	T	**166**	18.0		46.4	
MCT2				**0.048**		**< 0.001**
	NT	**132**	37.9		16.7	
	T	**166**	26.0		53.0	
MCT4				0.474		**< 0.001**
	NT	**128**	16.4		7.8	
	T	**168**	20.8		23.2	
CD147				0.211		0.153
	NT	**134**	11.9		41.1	
	T	**167**	16.7		32.9	

ISG, intermediate score group; HSG, high score group.

cancer. With this purpose, we analysed the expressions of MCT1, MCT2, MCT4, CD147 and gp70 in a series of prostate samples, including normal, adjacent non-neoplastic, PIN lesions and neoplastic tissues.

MCT1, MCT2, MCT4 and CD147 are differentially expressed in non-neoplastic, PIN lesions and neoplastic prostate tissues

In the present study, a general decrease in MCT1 and CD147 levels from normal prostate tissue to PIN lesions and prostate carcinoma was observed. Since their expression in normal prostate epithelium is high, it appears that they have an important role in normal tissue and are downregulated in prostate cancer cells, where other adaptive mechanisms may be activated. Importantly, there was an increase in both MCT2 and MCT4 expressions from normal to PIN lesions and tumour samples. In part, our results contradict the ones of Hao et al. [14], which stated that both MCT1 and MCT4 are upregulated in prostate cancer tissue, however the percentage of MCT4 positivity for pT3 tumours (around 40%) is similar to ours (44% tumour positive cases). Since the number of cases analysed by these authors is only slightly smaller than ours, the differences observed may be due to the different antibodies used. The specificity of our antibodies was tested by western-blot and more recently by RNAi for MCT1 (data not shown). We also observed frequent and clear MCT1 membrane expression, while these authors state that MCT1 staining was mostly cytoplasmic. The granular appearance of MCT2 and MCT4 expression in the cytoplasm of prostate tumour cells, as well as the predominance of the strong immunostaining scores suggests that these MCT isoforms could have important functions in some organelle membranes, possibly playing a role in the metabolism of prostate tumour cells.

Importantly, MCT2 was the only marker which allowed distinction between adjacent non-neoplastic tissue and PIN lesions. For MCT1, MCT4 and CD147, expression was similar in both. Despite apparently normal to the Pathologist, adjacent non-neoplastic glands already present alterations from normal tissue.

Monocarboxylate transporters 1 and 4 expression is associated with CD147 in prostate cancer

A close association between CD147 expression and both MCT1 and 4 was found, similar to the findings of Hao et al. [14]. Additionally, we detected no association with MCT2, supporting CD147 as chaperone for both MCT1 and MCT4 but not with MCT2 in prostate cancer. Studies of CD147 in paraffin-embedded specimens of prostate cancer are not many, however they describe overexpression of CD147 in prostate cancer, reporting expressions between 60 to 80% [22-25]. In the present study, we found around 50% of prostate tumour cases expressing CD147 and this expression was significantly different from the normal prostate tissue. To the best of our knowledge, our study has the biggest sample size and the number of non-neoplastic samples analysed is similar to the tumour samples.

Gp70 was only expressed in very few cancer cases and there was no association with either MCT isoforms. This result was surprising, however, since MCT2 was not present in the plasma membrane, the mechanism of regulation might be different. In addition, as described for CD147 and MCT isoforms 1 and 4 [31], there might be other chaperones involved in MCT2 regulation.

MCT4 and CD147 overexpression is associated with poor prognosis in prostate cancer

Assessment of associations between MCTs and CD147 expression and clinico-pathological data, revealed some

Table 2 Association between MCT1, MCT2, MCT4 and CD147 expressions in prostate tumours

	MCT1			*MCT4*			*MCT2*		
	n	Positive (%)	p	n	Positive (%)	p	n	Positive (%)	p
CD147			**< 0.001**			**< 0.001**			0.184
Negative	**59**	20.3		**84**	26.7		**35**	40.0	
Positive	**107**	64.5		**83**	63.0		**131**	50.4	

Table 3 Correlations between monocarboxylate transporters (MCTs) and CD147 expressions in prostate tumour samples and clinico-pathological data

Variable		MCT1				MCT2				MCT4				CD147			
	n	ISG % (n)	*p*	HSG %(n)	*p*	ISG % (n)	*p*	HSG %(n)	*p*	ISG % (n)	*p*	HSG %(n)	*p*	ISG % (n)	*p*	HSG %(n)	*p*
Age			0.391		0.073		**0.023**		0.117		0.247		< **0.001**		0.518		< **0.001**
> 64	**93**	19.4 (18)		40.9 (38)		32.6 (30)		48.4 (45)		18.5 (17)		8.6 (8)		17.2 (16)		16.1 (15)	
> 64	**73**	16.4 (12)		53.4 (39)		17.8 (13)		58.9 (43)		24.0 (18)		42.3 (31)		16.2 (12)		54.1 (40)	
PSA (ng/ml)			0.414		**0.016**		0.085		0.250		0.060		< **0.001**		0.529		< **0.001**
> 11	**116**	19 (22)		40.5 (47)		29.6 (34)		50.9 (116)		17.2 (20)		11.1 (13)		17.1 (20)		16.2 (19)	
> 11	**50**	16 (8)		60.0 (30)		18.0 (9)		58.0 (50)		29.4 (15)		51.0 (26)		16.0 (8)		72.0 (36)	
pT			0.511		0.145		0.528		0.297		0.210		< **0.001**		0.396		< **0.001**
2	**130**	18.5 (24)		43.8 (57)		26.4 (34)		51.5 (67)		19.2 (25)		14.5 (19)		16.0 (21)		24.4 (32)	
3	**36**	16.7 (6)		55.6 (20)		25.0 (3)		58.3 (21)		27.0 (10)		54.1 (20)		19.4 (7)		63.9 (23)	
Gleason score			0.251		0.170		0.386		0.857		0.692		**0.011**		0.850		**0.012**
< 7	**57**	15.8 (9)		52.6 (30)		31.6 (18)		54.4 (31)		24.6 (14)		10.5 (6)		15.8 (9)		21.1 (12)	
7	**100**	21.0 (21)		41.0 (41)		22.2 (22)		53.0 (53)		18.8 (19)		28.4 (29)		17.8 (18)		36.6 (36)	
> 7	**9**	0 (0)		66.7 (6)		33.3 (3)		44.4 (4)		22.2 (2)		44.4 (4)		11.1 (1)		66.7 (6)	
Perineural Invasion			0.525		**0.036**		0.503		0.259		0.397		**0.011**		0.531		**0.021**
Absent	**52**	17.3 (9)		57.7 (30)		26.9 (14)		57.7 (30)		23.1 (12)		11.5 (6)		17.3 (9)		21.2 (11)	
Present	**114**	18.4 (21)		41.2 (47)		25.7 (29)		50.9 (58)		20.0 (23)		28.4 (33)		16.5 (19)		38.3 (44)	
Biochemical Recurrence			0.434		**0.047**		0.597		0.089		0.200		< **0.001**		0.489		< **0.001**
Absent	**139**	18.7 (26)		43.2 (60)		26.1 (36)		50.4 (70)		19.4 (27)		17.1 (24)		16.4 (23)		26.4 (37)	
Present	**27**	14.8 (4)		63.0 (17)		25.9 (7)		66.7 (18)		28.6 (8)		53.6 (15)		18.5 (5)		66.7 (18)	

ISG, intermediate score group; HSG, high score group.

important associations. In accordance to the work of other groups, we found positive associations between both MCT4 and CD147 expressions and higher PSA levels, advanced tumour stage and higher Gleason score [14,22-25]. However, regarding tumour stage, the difference in MCT4 expression in the report of Hao and collaborators [14], appears to be only from pT1 to pT2 and 3 since the levels of the last two appear to be similar. In our series we do not have pT1 stage cases, which makes the comparison more difficult. Additionally, we found associations between MCT1 expression and higher PSA levels, absence of perineural invasion and

presence of biochemical recurrence, as well as between both MCT4 and CD147 and presence of perineural invasion and biochemical recurrence, which to the best of our knowledge, was not described by others. MCT2 also correlated with lower age at diagnosis, while both MCT4 and CD147 were associated with higher age at diagnosis, which significance is not known.

The differences observed between our study and others, besides the use of different antibodies, might be also due to the diverse immunohistochemical assessment, while in the present study we considered both intensity and percentage of immunoreactive cells, other authors considered only either intensity or extension of staining [14,22-25]. We believe that evaluating two parameters instead of one, decreases subjectivity and will have higher biological significance. Moreover, our non-neoplastic tissue was selected from the peripheral zone of the prostate in which prostate cancer is diagnosed and this may also have contributed to the differences observed.

Overall, we found no important clinicopathological associations with MCT2 but tumours that are highly positive (HSG) for MCT1, MCT4 or CD147, seem to exhibit a more aggressive behaviour, especially MCT4 and CD147 which correlated with higher PSA levels, higher pT stage, higher Gleason score, presence of perineural invasion and biochemical recurrence. However, to elucidate the significance of these associations, functional studies will be needed.

MCTs and prostate cancer metabolism

At variance with other solid tumours [6,7,9], we did not find up-regulation of MCT1, MCT4 or CD147 in the plasma membrane of prostate cancer cells, proteins normally involved in the hyper glycolytic-acid resistant phenotype of cancer cells. On the other hand, there was upregulation of MCT2 and MCT4 in the cytoplasm of cancer cells, with a granular appearance. These findings suggest two hypothesis: either presence of alternative mechanisms that ensure acid efflux and maintenance of intracellular pH, or presence of an alternative metabolic pathway different from glycolysis that predominates in prostate cancer. Indeed, β-oxidation pathway is suggested to be up-regulated in prostate cancer [32] and α-methylacyl-CoA racemase (AMACR), an enzyme involved in branched chain fatty acid β-oxidation, is already being explored as a diagnostic marker of prostate cancer [33]. Additionally, MCT2, analysed for the first time in the present work, is involved in short-chain fatty acid transport and appears overexpressed and with strong staining in the cytoplasm of prostate tumour cells, with a granular appearance. These facts point to this MCT isoform as an important protein in prostate tumour cells, likely involved in some organelle function. In fact, the work of McClelland and collaborators provide evidence for a putative role of MCT2 in hepatocyte peroxisomal membrane [34].

Conclusion

In the present study, we analysed the expression of MCT1, MCT2, MCT4, CD147 and gp70, in prostate cancer, corresponding adjacent non-neoplastic tissue, normal tissue and PIN lesions, and sought for associations with the clinico-pathological data of the patients. Our data provides novel evidence for the involvement of MCTs in prostate tumours. According to our results, we believe that MCT2 should be further explored as tumour marker and MCT4 and CD147 as markers of poor prognosis in prostate cancer.

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Authors' contributions

FB, JRV and CL were responsible for the study concept and design, study supervision, manuscript drafting and critical revision. NPG, VMG, CP, HP and JS performed the immunohistochemistry reactions and participated in the drafting of the manuscript. NPG, JRV, RMH were responsible for sample and clinic pathological collection and JRV and PM evaluated the immunohistochemical reactions.

All the authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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References

1. Warburg O, Wind F, Neglein E: **Metabolism of tumours in the body.** *J Gen Physiol* 1927, **8**:519-530.
2. Gatenby RA, Gillies RJ: **Why do cancers have high aerobic glycolysis?** *Nat Rev Cancer* 2004, **4**:891-899.
3. Izumi H, Torigoe T, Ishiguchi H, Uramoto H, Yoshida Y, Tanabe M, Ise T, Murakami T, Yoshida T, Nomoto M, Kohno K: **Cellular pH regulators: potentially promising molecular targets for cancer chemotherapy.** *Cancer Treat Rev* 2003, **29**:541-549.
4. Semenza GL, Artemov D, Bedi A, Bhujwalla Z, Chiles K, Feldser D, Laughner E, Ravi R, Simons J, Taghavi P, Zhong H: **The metabolism of tumours: 70 years later.** *Novartis Found Symp* 2001, **240**:251-260.

5. Halestrap AP, Price NT: **The proton-linked monocarboxylate transporter MCT. Family: structure, function and regulation.** *Biochem J* 1999, **343**:281-299.
6. Pinheiro C, Longatto-Filho A, Scapulatempo C, Ferreira L, Martins S, Pellerin L, Rodrigues M, Alves VA, Schmitt F, Baltazar F: **Increased expression of monocarboxylate transporters 1, 2 and 4 in colorectal carcinomas.** *Virchows Arch* 2008, **452**:139-146.
7. Pinheiro C, Longatto-Filho A, Ferreira L, Pereira S, Etlinger D, Moreira M, Jubé L, Queiroz G, Schmitt F, Baltazar F: **Increasing expression of monocarboxylate transporters 1 and 4 along progression to invasive cervical carcinoma.** *Int J Gynec Pathol* 2008, **27**:568-574.
8. Wahl ML, Owen JA, Burd R, Herlands RA, Nogami S, Rodeck U, Berd D, Leeper DB, Owen CS: **Regulation of intracellular pH in human melanoma: potential therapeutic implications.** *Mol Cancer Ther* 2002, **37**:617-628.
9. Pinheiro C, Albergaria A, Paredes J, Sousa B, Dufloth R, Vieira D, Schmitt F, Baltazar F: **Monocarboxylate transporter 1 is up-regulated in basal-like breast carcinoma.** *Histopathology* 2010, **56**:860-867.
10. Pinheiro C, Reis RM, Ricardo S, Longatto-Filho A, Schmitt F, Baltazar F: **Expression of monocarboxylate transporters 1, 2, and 4 in human tumours and their association with CD147 and CD44.** *J Biomed Biotechnol* 2010, **2010**:427694, Epub 2010 May 4.
11. Koukourakis MI, Giatromanolaki A, Bougioukas G, Sivridis E: **Lung cancer: a comparative study of metabolism related protein expression in cancer cells and tumour associated stroma.** *Cancer Biol Ther* 2007, **6**:1476-1479.
12. Gomes N, Vizcaíno JR, Pinheiro C, Baltazar F: **Monocarboxylate transporters 2 and 4 are upregulated in prostate carcinoma.** *Virchows Arch* 2009, **455(Supplement: Suppl. 1)**:233.
13. Gomes N, Vizcaíno JR, Pinheiro C, Baltazar F: **Characterization of monocarboxylate transporters expression in prostate carcinoma.** *Microsc Microanal* 2009, **15(Supplement S3)**:17-18.
14. Hao J, Chen H, Madigan MC, Cozzi PJ, Beretov J, Xiao W, Delprado WJ, Russell PJ, Li Y: **Co-expression of CD147 (EMMPRIN), CD44v3-10, MDR1 and monocarboxylate transporters is associated with prostate cancer drug resistance and progression.** *Br J Cancer* 2010, **103(7)**:1008-1018.
15. Kirk P, Wilson MC, Heddle C, Brown MH, Barclay AN, Halestrap AP: **CD147 is tightly associated with lactate transporters MCT1 and MCT4 and facilitates their cell surface expression.** *EMBO J* 2000, **19**:3896-3904.
16. Wilson MC, Meredith D, Fox JE, Manoharan C, Davies AJ, Halestrap AP: **Basigin (CD147) is the target for organomercurial inhibition of monocarboxylate transporter isoforms 1 and 4: the ancillary protein for the insensitive MCT2 is EMBIGIN (gp70).** *J Biol Chem* 2005, **280**:27213-27221.
17. Deora AA, Philp N, Hu J, Bok D, Rodriguez-Boulan E: **Mechanisms regulating tissue-specific polarity of monocarboxylate transporters and their chaperone CD147 in kidney and retinal epithelia.** *Proc Natl Acad Sci USA* 2005, **102**:16245-16250.
18. Gallagher SM, Castorino JJ, Wang D, Philp NJ: **Monocarboxylate transporter 4 regulates maturation and trafficking of CD147 to the plasma membrane in the metastatic breast cancer cell line MDA-MB-231.** *Cancer Res* 2007, **67**:4182-4189.
19. Nabeshima K, Iwasaki H, Koga K, Hojo H, Suzumiya J, Kikuchi M: **Emmprin (basigin/CD147): matrix metalloproteinase modulator and multifunctional cell recognition molecule that plays a critical role in cancer progression.** *Pathol Int* 2006, **56**:359-367.
20. Gabison EE, Hoang-Xuan T, Mauviel A, Menashi S: **EMMPRIN/CD147, an MMP modulator in cancer, development and tissue repair.** *Biochimie* 2005, **87**:361-368.
21. Riethdorf S, Reimers N, Assmann V, Kornfeld JW, Terracciano L, Sauter G, Pantel K: **High incidence of EMMPRIN expression in human tumors.** *Int J Cancer* 2006, **19**:1800-1810.
22. Zhao-Dong H, Xue-Cheng B, Wei-jun Q, He H, Dai Q, Zou J, Ye Y, Liang Y, Zeng G, Chen Z, Zhong W: **CD147 Expression indicates unfavourable prognosis in prostate cancer.** *Pathol Oncol* 2009, **15**:369-374.
23. Zhong WD, Han ZD, He HC, Bi XC, Dai QS, Zhu G, Ye YK, Liang YX, Qin WJ, Zhang Z, Zeng GH, Chen ZN: **CD147, MMP-1, MMP-2 and MMP-9 protein expression as significant prognostic factors in human prostate cancer.** *Oncology* 2008, **75**:230-236.
24. Madigan MC, Kingsley EA, Cozzi PJ, Madigan MC, Kingsley EA, Cozzi PJ, Delprado W, Russel P, Li Y: **The role of extracellular matrix metalloproteinase inducer protein in prostate cancer progression.** *Cancer Immunol Immunother* 2008, **57**:1367-1379.
25. Han ZD, He HC, Bi XC, Qin WJ, Dai QS, Zou J, Ye YK, Liang YX, Zeng GH, Zhu G, Chen ZN, Zhong WD: **Expression and clinical significance of CD147 in genitourinary carcinomas.** *J Surg Res* 2010, **160**:260-267.
26. Epstein J, Allsbrook W, Amin M, Egevad LL, ISUP Grading Committee: **The 2005 International Society of Urological Pathology (ISUP) Consensus conference on gleason grading of prostatic carcinoma.** *Am J Sur Pathol* 2005, **29**:1228-1242.
27. Edge SB, Byrd DR, Compton CC, Fritz AG, Greene FL, Trotti A: *AJCC cancer staging manual*. 7 edition. Chicago:Springer; 2010.
28. Damber JE, Aus G: **Prostate cancer.** *Lancet* 2008, **371**:1710-1721.
29. Mathupala SP, Parajuli P, Sloan AE: **Silencing of monocarboxylate transporters via small interfering ribonucleic acid inhibits glycolysis and induces cell death in malignant glioma: an in vitro study.** *Neurosurgery* 2004, **55**:1410-1419.
30. Sonveaux P, Vegran F, Schroeder T, Wergin M, Verrax J, Rabbani ZN, Saedeleer CJ, Kennedy KM, Diepart C, Jordan BF, Kelley MJ, Gallez B, Wahl ML, Feron O, Dewhirst MW: **Targeting lactate-fueled respiration selectively kills hypoxic tumor cells in mice.** *J Clin Invest* 2008, **118**:3930-3942.
31. Slomiany MG, Grass GD, Robertson AD: **Hyaluronan, CD44, and emmprin regulate lactate efflux and membrane localization of monocarboxylate transporters in human breast carcinoma cells.** *Cancer Res* 2009, **69**:1293-1301.
32. Legaspi A, Jeevanandam M, Starnes HF Jr, Brennan MF: **Whole body lipid and energy metabolism in the cancer patient.** *Metabolism* 1987, **36**:958-963.
33. Luo J, Zha S, Gage WR, Dunn TA, Hicks JL, Bennett CJ, Ewing CM, Platz EA, Ferdinandusse S, Wanders RJ, Trent JM, Isaacs WB, De Marzo AM: **α-Methylacyl-CoA racemase: a new molecular marker for prostate cancer.** *Cancer Res* 2002, **62**:2220-2226.
34. McClelland G, Khanna S, Brooks G: **Peroxisomal membrane monocarboxylate transporters: evidence for a redox shuttle system?** *Biochem Biophys Res Commun* 2003, **304**:130-135.

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CHAPTER 3. MONOCARBOXYLATE TRANSPORTER 2 (MCT2) IN PROSTATE CANCER: EXPRESSION, FUNCTION AND EXPLOITATION AS A BIOMARKER

3.1 CHAPTER OVERVIEW

Besides the challenges in the prognostic and therapeutic field in PCa, the diagnosis of the disease can also represent a challenge.

The diagnosis of prostate cancer can sometimes present a diagnostic challenge for pathologists, since, prostate carcinoma can mimic benign prostate glands and the architectural or cytologic clues to a diagnosis of carcinoma may not always be seen in small foci of suspicious glands. Also, diagnosis of prostate cancer can be difficult in needle biopsies or in minimal residual cancer of radical prostatectomies, presenting one of the major challenges in surgical pathology. Therefore, it would be of great importance and usefulness to have molecular markers with high sensitivity and specificity for prostate carcinoma.

In the previous chapter we observed an evident increase in MCT2 expression in the cytoplasm of PCa cells comparing to non-neoplastic tissue. This observation lead us to explore more deeply MCT2 as a putative prostate cancer biomarker. In this chapter we present results where we compare the sensitivity and specificity of MCT2 and AMACR (an already established prostate cancer biomarker) in recognizing prostate cancer, by analyzing the immunohistochemical expression of both markers in a large series of prostate samples, including normal prostate, non-neoplastic tissue, PIN lesions and tumour tissues and assess its clinico-pathological value. Also, we aimed to measure the sensitivity and specificity of combining MCT2 and AMACR as positive markers together with the negative markers p63 and 34βE12, using tissue microarrays (TMAs), which recapitulate the small problematic foci of glandular proliferation that are generally encountered in prostatic biopsy specimens. Also, we aimed to study the subcellular localization of MCT2 in prostate cancer cells across malignant transformation.

Our study revealed that MCT2 as well as AMACR is consistently expressed in prostate cancer regardless Gleason score and in combination with AMACR and p63 or 34βE12 will help to improve the diagnosis of prostate carcinoma. Importantly, we found MCT2 interacting with PEX19 and co-localizing with peroxisomal clusters, being this co-localization more evident from the non-malignant model PNT1a to the malignant 22RV1, suggesting once again a role for

MCT2 in prostate malignant transformation, more than in the aggressiveness and progression of the disease.

3.2 PUBLISHED RESULTS

The results presented in this chapter were:

(i) Published as an original article in an international peer reviewed journal

Pértega-Gomes N. Vizcaíno JR, Gouveia C., Jerónimo C., Henrique RM., Lopes C. and Baltazar F. et al "Monocarboxylate transporter 2 (MCT2) as putative biomarker in prostate cancer. **The Prostate.** 2012 Nov 28. doi: 10.1002/pros.22620.

(ii) Submitted for publication as an original article in an international peer reviewed journal

Pértega-Gomes N, Valença I, Vizcaíno JR, Ribeiro D., Baltazar F. "Localization of MCT2 at peroxisomes is associated with malignant transformation in prostate cancer"2013.

(iii) Presented as oral communication in the following national scientific meeting:

Pértega-Gomes N, Vizcaino JR, Gouveia C, Lopes C and Baltazar F. 2010 "Comparative study between MCT2 and AMACR in prostate cancer diagnosis" Work presented at Pathology Department Day at Centro Hospitalar do Porto (CHP).

(iv) Presented as poster in the following national scientific meeting:

Pértega-Gomes N, Vizcaino JR, Gouveia C, Lopes C and Baltazar F. 2010. "Monocarboxylate transporter 2 as a putative prostate cancer biomarker" at XIX Porto Cancer Meeting at IPATIMUP, Porto, Portugal.

(v) *Were recognized with the following prizes/awards:*

Pértega-Gomes N., *Vizcaino JR., Gouveia C., Lopes C. and Baltazar F. 2010 "Comparative study between MCT2 and AMACR in prostate cancer diagnosis"* ***1st Prize for best poster and oral communication (Dr.Mário Costa Award) in Laboratorial*** **Pathology meeting of CHP-Hospital de Sto.António, Porto, Portugal.**

3.2.1 Monocarboxylate transporter 2 (MCT2) as a putative biomarker in prostate cancer.

Monocarboxylate Transporter 2 (MCT2) as Putative Biomarker in Prostate Cancer

Nelma Pértega-Gomes,[1,2] José R. Vizcaíno,[3] Carlos Gouveia,[3] Carmen Jerónimo,[4,5,6] Rui M. Henrique,[4,6,7] Carlos Lopes,[3,6] and Fátima Baltazar[1,2]*

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BACKGROUND. Monocarboxylate transporter 2 (MCT2) is a transmembrane protein involved in the transport of monocarboxylates such as pyruvate and lactate. In a previous study we described overexpression of MCT2 in prostate carcinoma raising the hypothesis of using MCT2 as a possible biomarker in prostate cancer.
With the present study we aimed to compare the pattern of expression of MCT2 and alpha-methylacyl-CoA racemase (AMACR), in prostate carcinoma, PIN lesions, non-neoplastic prostate tissue, and normal prostate and compare their sensitivity and specificity. Also, we wanted to evaluate the value of using MCT2 in combination with AMACR and the negative markers 34βE12 or p63 to detect prostate cancer.
METHODS. A total of 349 cases, including prostate carcinoma, non-neoplastic prostate tissue and PIN lesions, from radical prostatectomies were examined by immunohistochemistry for AMACR, MCT2, p63, and 34βE12, using tissue microarrays (TMAs). Normal prostate from radical cystoprostatectomy was also studied.
RESULTS. Our study revealed that MCT2, similarly to AMACR, was consistently expressed in prostate cancer regardless of the Gleason score. In combination with AMACR and p63 or 34βE12, MCT2 helped to improve the diagnosis of prostate carcinoma. Also, overexpression of MCT2 as well as AMACR in PIN lesions may indicate the involvement of these two proteins in prostate cancer initiation.
CONCLUSIONS. We provided evidence for the presence of MCT2 in prostate cancer, selectively labeling malignant glands. Importantly, assessment of MCT2 together with AMACR, along with the negative markers, highly increases the accuracy in prostate cancer diagnosis.
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KEY WORDS: **alpha-methylacyl-CoA racemase; cancer biomarkers; prostate cancer diagnosis**

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he authors declare that they have no competing interests.

thics: The present study was approved by the local Ethic ommittees.

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INTRODUCTION

Worldwide, prostate cancer is the second most common malignancy in men after lung cancer [1]. Diagnosis of prostate cancer glands can sometimes present a diagnostic challenge for pathologists, since, prostate carcinoma can mimic benign prostate glands [2] and the architectural or cytologic clues for the diagnosis of carcinoma may not always be seen in small foci of suspicious glands. Also, diagnosis of prostate cancer can be difficult in needle biopsies or in minimal residual cancer of radical prostatectomies, presenting one of the major challenges in surgical pathology. Underdiagnosis of a small focus of prostatic adenocarcinoma or overdiagnosis of a benign lesion mimicking cancer is not uncommon and can cause unfortunate consequences for patients and is a liability for pathologists. Therefore, it would be of great importance and usefulness to identify molecular markers with high sensitivity and specificity for prostate carcinoma.

Monocarboxylate transporters (MCTs) are transmembrane proteins which facilitate the membrane transport of important monocarboxylates, such as pyruvate and lactate. In glycolytic tumors, they promote the efflux of lactic acid, being important players in the maintenance of the tumor intracellular pH [3,4]. In a first study we assessed the immunoexpression of MCTs 1, 2, 4 and their protein chaperones in a well characterized prostate carcinoma series [5]. This study revealed a significant increase of MCT2 expression in tumor cells with a predominance of the strong score, which means that we could distinguish between MCT2 expression in tumor tissue from the expression of this protein in non-neoplastic tissue.

In the same study [5], we noted that MCT2 staining was very similar to AMACR (alpha-methylacyl-CoA racemase), an enzyme currently used in prostate cancer diagnosis, which is a peroxisomal and mitochondrial enzyme that was found to be up-regulated in prostate cancer [6–8]. AMACR plays an important role in bile acid biosynthesis and β-oxidation of branched-chain fatty acids and mediates the interconversion of (R) and (S)-2-methyl-branched-chain fatty acyl-CoAs [9,10], however, the biological significance of its expression in tumorigenesis is still not elucidated.

Some studies suggested that the use of AMACR as a positive marker alone may be misleading since expression of AMACR might be seen in benign glands, and non-malignant lesions [11]. Therefore, other studies report the use of AMACR as a positive marker along with the basal cell-specific negative markers 34βE12 and p63, which are absent in the vast majority of prostate carcinomas, to enhance the diagnostic accuracy and reduce the chance of misdiagnosis [12]. 34βE12 is a high-molecular-weight cytokeratin that is expressed in the cytoplasm of basal cells rather than in luminal or secretory cells [13]; p63 has selective expression in the basal cell compartment of various epithelial tissues and has high sensitivity in identifying the nuclei of basal cells in benign prostatic lesions [14–16].

This study aims to compare the sensitivity and specificity of MCT2 and AMACR in recognizing prostate cancer, by analyzing the immunohistochemical expression of both markers in a large series of prostate samples, including normal prostate, adjacent non-neoplastic tissue, PIN lesions and tumor tissues, and assess their clinico-pathological value. Also, we aimed to measure the sensitivity and specificity of combining MCT2 and AMACR as positive markers with the negative markers p63 and 34βE12, using tissue microarrays (TMAs), which recapitulate the small problematic foci of glandular proliferation that are generally encountered in prostatic biopsy specimens.

MATERIALS AND METHODS

Case Selection and Tissue Microarray Construction

Prostate samples were obtained from 349 patients with prostate carcinoma (including adjacent non-neoplastic tissues, PIN lesions, and primary tumors), with a median age of 64 years (range 46–74) selected from a cohort of patients who underwent radical prostatectomy in Centro Hospitalar do Porto—Portugal as a primary therapy (no preceding hormonal or radiation therapy) for clinically localized prostate cancer between 1993 and 2010. Benign prostate tissue was obtained from cystoprostatectomy specimens.

TMAs were constructed as previously reported [5]. Tumors were staged using the 2010 pTNM AJCC classification [17], which includes extra-prostatic extension and graded using the Gleason grading system 2005 [18].

Although there is no universal method of sampling prostate cancer tissue for immunohistochemistry slides, using either standard slides or TMAs, the histological features of the sampled areas that we sampled were representative of the final Gleason score for the case.

Immunohistochemistry

MCT2, AMACR, p63, and 34βE12 detection. Immunohistochemistry for MCT2, AMACR, p63, and 34βE12 was performed according to avidin–biotin–peroxidase complex principle with the primary

antibody for MCT2 (sc-14926, Santa Cruz Biotechnology, Santa Cruz, CA), AMACR (504R-16, Cell Marque), p63 (MS-1084-P, Neomarkers), and 34βE12 (334M-8, Cell Marque) diluted 1:200, 1:50, 1:100, and 1:100, respectively. Negative controls were performed by omitting of the primary antibody. Normal kidney was used as positive control for MCT2, AMACR, and p63. Human skin was used as 34βE12 positive control.

Tissue sections were counterstained with hematoxylin and permanently mounted.

Immunohistochemical Evaluation

All samples were scored for AMACR and MCT2 protein expression intensity. Protein expression was scored as negative (score = 0), weak (score = 1), moderate (score = 2), or strong (score = 3). Moderate or strong staining intensity was considered positive (score = 2 or 3) as previously described for AMACR [19].

Each case positive for AMACR and MCT2 was also evaluated for the percentage of glands/cells that stained for AMACR and MCT2 and scored as: <5%, 5–50%, and >50%.

Positive immunohistochemical staining for 34βE12 and p63 was defined as nuclear reactivity for p63 and cytoplasmic positivity for 34βE12 [12,20].

Criteria for interpretation of the antibody combination were as follows: cases were considered true-positive when 34βE12/p63 and AMACR, all of the three antibodies stained as for a malignant lesion, that is, 34βE12 and p63 stains absent and AMACR present. Using the combination with p63/AMACR/MCT2 or 34βE12/AMACR/MCT2 true-positive was considered when p63 and 34βE12 stains were absent but AMACR and/or MCT2 stains were present [20].

Each reaction was observed by two experienced pathologist (J.R.V. and C.L.), without prior knowledge of associated clinical or pathology staining information. Discordant results were discussed in a double-head microscope (J.R.V. and C.L.).

Statistical Analysis

Statistical analysis was performed using the SPSS statistical software (version 17.0, SPSS Inc., Chicago, IL). All comparisons were examined for statistical significance using Pearson's chi-square (χ^2) test, being the threshold for significance $P < 0.05$.

RESULTS

Prostate samples were organized into TMAs, including 349 neoplastic tissues, 40 PIN lesions, 203 non-neoplastic, and 13 normal prostate cases from cystoprostatectomy were analyzed for MCT2, AMACR, 34βE12, and p63 immunohistochemical expression.

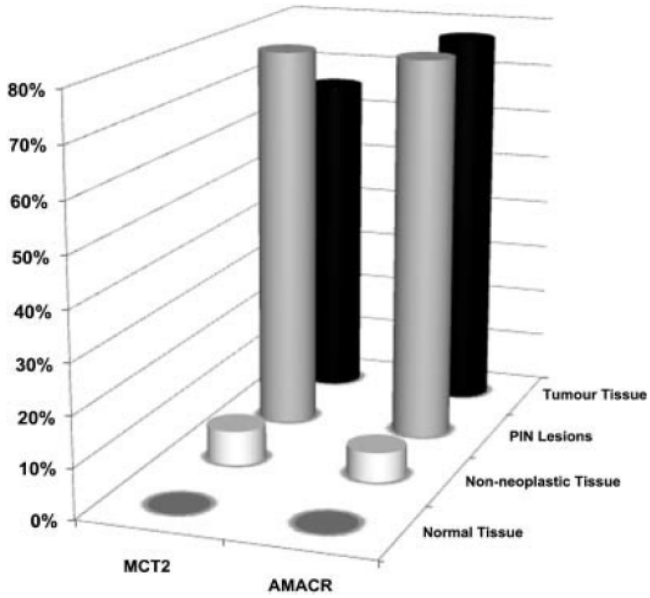

Fig. 1. Frequency of MCT2 and AMACR expressions in normal prostate tissue, non-neoplastic tissue, PIN lesions, and tumor tissue samples.

Figure 1 summarizes MCT2 and AMACR expressions in normal prostate, adjacent non-neoplastic tumor tissue, PIN lesions and primary tumor tissues. For the expression of both markers, AMACR and MCT2, we can observe a significant increase in tumor tissues and PIN lesions compared to normal prostate and non-neoplastic tissue samples ($P < 0.001$). Normal prostate samples were both negative for MCT2 and AMACR expressions. From 203 non-neoplastic samples, 14 stained for MCT2 (6.9%) and 12 for AMACR (5.9%). Both proteins showed overexpression in PIN lesions (77.5% for both). From the 349 malignant cases, 230 (65.9%) stained for MCT2 and 270 (77.4%) for AMACR.

Figure 2 shows representative immunohistochemical reactions for MCT2, AMACR, p63, and 34βE12 in normal prostate tissue (Fig. 2A, D, G, and J), PIN lesions (Fig. 2B, E, H and K, respectively), and tumor (Fig. 2C, F, I and L, respectively). The staining observed for MCT2 was very similar to that observed for AMACR. In most cases, adenocarcinomas showed moderate to strong staining in the majority of tumor cells for both proteins. Expression was uniformly cytoplasmic with a typical fine granular pattern. Nuclear staining of p63 and cytoplasmic staining for 34βE12 were confined to basal cells in normal prostate tissue (Fig. 2G and J, respectively).

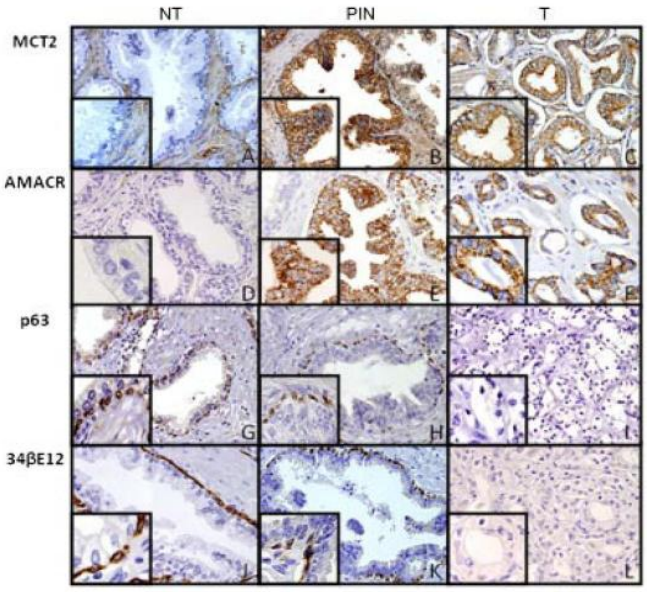

Fig. 2. Immunohistochemical expression of MCT2 (**A**–**C**), AMACR (**D**–**F**), p63 (**G**–**I**), and 34βEI2 (**J**–**L**) in normal prostate tissue (NT), PIN lesions (PIN), and prostate tumor tissues (T). Main pictures are at 200× magnification and insets are at 400×.

To assess if the staining extension pattern of MCT2 and AMACR in tumor tissues is associated with Gleason score, we compared the extension distribution of MCT2 and AMACR positivity in all Gleason grades (Tables I and II, respectively). We noted that regardless Gleason score, the diffuse overexpression (>50% of tumor stained) of AMACR and MCT2 predominated and was observed in 65.5% and 57.4% of the cases, respectively.

Assessment of associations between MCT2 and AMACR expressions and the clinico-pathological data revealed no significant associations with Gleason score, pathological stage, patients' age, or preoperative serum specific antigen, perineural invasion, or biochemical recurrence (data not shown) as it was already observed for MCT2 using a different scoring methodology [5].

Specificity and sensitivity to detect tumor were calculated for the markers individually (Table III), as well as within the triple combinations (Table IV). From the 349 malignant samples stained, observing the different antibodies individually, 349 (100%) did not stain for 34βE12 or p63, 230 (65.9%), and 270 (77.4%) were positive for MCT2 and AMACR, respectively (Table III). From 203 benign prostatic TMA samples stained, 189 (93.1%) stained for 34βE12 and 191 (96.7%) stained for p63. For AMACR and MCT2, 191 (94.1%) cases and 189 (93.1%) benign samples were negative for AMACR and MCT2, respectively (Table III).

From the 349 malignant prostatic samples classified as malignant lesions, 270 (77.4%) stained negatively for 34βE12 and p63 and positively for AMACR (Table IV). Using the criteria of one negative marker (34βE12) plus two positive markers (AMACR/MCT2)

TABLE I. Extension of MCT2 Expression in Prostate Cancer Relative to Gleason Score

			Extension of the staining		
Gleason score	No. of cases	Positive cases	<5%+	5% to 50%+	>50%+
≤5	3	2 (0.8%)	0	0	2
6	83	55 (23.9%)	18	18	19
7	246	162 (70.4%)	23	35	104
≥8	17	11 (4.9%)	2	2	7
Total	349	230 (100%)	43 (18.7%)	55 (23.9%)	132 (57.4%)

TABLE II. Extension of AMACR Expression in Prostate Cancer Relative to Gleason Score

			Extension of the staining		
Gleason score	No. of cases	Positive cases	<5%+	5% to 50%+	>50%+
≤5	3	3 (1.1%)	0	1	2
6	83	62 (23%)	3	16	43
7	246	196 (72.6%)	21	52	123
≥8	17	9 (3.3%)	0	0	9
Total	349	270 (100%)	24 (8.9%)	69 (25.6%)	177 (65.5%)

TABLE III. Sensitivity and Specificity for the Antibodies Evaluated Separately

	34βE12	p63	AMACR	MCT2	Total
True positive (malignant Cases)	349	349	270	230	349
True negative (benign cases)	189	191	191	189	203
Sensitivity (%)	100	100	77.4	65.9	—
Specificity (%)	93.1	94.1	94.1	93.1	—
Positive predictive value (%)	96.1	96.7	95.7	94.3	—
Negative predictive value (%)	100	100	70.7	61.4	—

to identify a malignant lesion we have 327 of 349 malignant cases (87.7%) staining like a malignant lesion. The same results were obtained when using p63 as negative marker, that is, 87.7% of malignant cases staining like a true malignant case.

We also calculated the specificity, positive predictive value, and negative predictive value of the three combinations. We observed that all have a specificity and positive predictive value of 95.9% and 96.5% but with the use of two negative markers and only one positive marker we obtain a negative predictive value of 69.3% whereas with both AMACR and MCT2 as positive markers and only one negative marker, p63 or 34βE12, we can obtain a negative predictive value of 89.6% or 89.7%, respectively (Table IV).

DISCUSSION

With the major effort to early detect prostate cancer by men mass screening, there have been an increasing number of small foci of cancer encountered in prostate specimens. Inconclusive images on standard H&E staining are occasionally encountered, implying but not confirming the presence of malignancy. Such findings are often described as ''atypical foci'' and in most cases dictate a second biopsy [21–23].

It has been shown that using AMACR as a positive marker in association with the traditional basal cell-specific 34βE12 and/or p63 as negative markers can help to confirm the diagnosis when small atypical glands are identified by routine H&E staining [12,20]. It becomes more evident that it is crucial to use a combination of positive and negative markers for immunohistochemical analysis to assist in the diagnosis of prostate cancer.

In the present study we analyzed the pattern of expression of MCT2 and AMACR in a large number of prostate cancer and benign prostate tissues to compare the sensitivity and specificity of MCT2 to detect prostate cancer when compared to AMACR, an already established prostate cancer biomarker. Immunohistochemistry revealed that like AMACR, MCT2 is overexpressed in the majority of prostate cancer cases with diverse pathologic characteristics. This overexpression occurs in virtually all Gleason grades with a predominancy of diffuse overexpression, with more than 50% of tumor stained in positive cases, meaning that the positivity of MCT2 as well as AMACR is independent of the Gleason score, which is in accordance with the data reported for AMACR [19].

Expression of MCT2 was comparable with AMACR, allowing prostate cancer diagnosis in a minimal amount of tissue, giving few false-negative/positive data.

When we observe the results of immunohistochemical staining for the antibodies evaluated individually, we noted that 34βE12 and p63 were the most sensitive and specific markers to distinguish prostate cancer, with 100% sensitivity for both and 93.1% and 94.1% of specificity for 34βE12 and p63, respectively. However, they are negative markers and there are many limitations of using negative markers alone for the

TABLE IV. Sensitivity and Specificity for the Antibodies Evaluated in Triple Combinations

	34βE12−/p63−/AMACR+	34βE12−/MCT2 and/or AMACR+	p63−/MCT2 and/or AMACR+	Total
True positive (malignant cases)	270	327	327	349
True negative (benign cases)	179	189	191	203
Sensitivity (%)	77.4	87.7	87.7	—
Specificity (%)	88.2	93.1	94.1	—
Positive predictive value (%)	91.8	95.9	96.5	—
Negative predictive value (%)	69.3	89.6	89.7	—

diagnosis of carcinoma, such as the fact that basal cells could be patchy or discontinuous in some benign lesions and lead to misdiagnosis. Consequently, negative staining for p63 or 34βE12 in a few glands suggestive of cancer is not proof of their malignancy since benign glands might not show uniform positivity with these markers.

Analyzing the results of immunohistochemical staining for the antibodies evaluated in the triple combination, we observed that the use of two positive markers with one negative marker instead of one positive marker with two negative markers improves the sensitivity to detect prostate cancer as well as the negative predictive value, which was 69.3% using the 34βE12/p63/AMACR combination and 89.6% or 89.7% when using 34βE12/MCT2 and/or AMACR or p63/MCT2 and/or AMACR. This result means that with these combinations of markers, we decrease the possibility of diagnosing benign prostate tissue as prostate cancer.

The observation that similarly to AMACR, MCT2 also stains strongly in PIN lesions, if on one hand decreases the specificity of these proteins, on the other hand indicates that these two proteins may be involved in tumor initiation. However, further studies are needed to clarify the role of both markers on prostate cancer initiation/progression.

CONCLUSIONS

Our study points to the consistent overexpression of MCT2 in prostate cancer, which is comparable to AMACR, an already established biomarker in prostate cancer. Importantly, assessment of MCT2 together with AMACR, along with the negative markers p63 and 34βE12, highly increases the accuracy in prostate cancer diagnosis.

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REFERENCES

1. Ferlay J, Bray F, Pisani P. Globocan 2002: Cancer incidence, mortality and prevalence worldwide. Version 2.0. Lyon, France: IARC Press; 2004. IARC CancerBase No. 5.
2. Gaudin PB, Reuter VE. Benign mimics of prostatic adenocarcinoma on needle biopsy. Anat Pathol 1997;2:111–134.
3. Vander Heiden MG. Targeting cancer metabolism: A therapeutic window opens. Nat Rev Drug Discov 2011;10(9):671–684.
4. Pinheiro C, Longatto-Filho A, Azevedo-Silva J, Casal M, Schmitt FC, Baltazar F. Role of monocarboxylate transporters in human cancers: State of the art. J Bioenerg Biomembr 2012;44(1):127–139.
5. Pértega-Gomes N, Vizcaíno JR, Miranda-Gonçalves V, Pinheiro C, Silva J, Pereira H, Monteiro P, Henrique RM, Reis RM, Lopes C, Baltazar F. Monocarboxylate transporter 4 (MCT4) and CD147 overexpression is associated with poor prognosis in prostate cancer. BMC Cancer 2011;11:312.
6. Dhanasekaran SM, Barrette TR, Ghosh D, Shah R, Varambally S, Kurachi K, Pienta KJ, Rubin MA, Chinnaiyan AM. Delineation of prognostic biomarkers in prostate cancer. Nature 2001;412:822–826.
7. Luo J, Duggan DJ, Chen Y, Sauvageot J, Ewing CM, Bittner ML, Trent JM, Isaacs WB. Human prostate cancer and benign prostatic hyperplasia: Molecular dissection by gene expression profiling. Cancer Res 2001;61:4683–4688.
8. Welsh JB, Sapinoso LM, Su AI, Kern SG, Wang-Rodriguez J, Moskaluk CA, Frierson HF Jr, Hampton GM. Analysis of gene expression identifies candidate markers and pharmacological targets in prostate cancer. Cancer Res 2001;61:5974–5978.
9. Ferdinandusse S, Denis S, IJlst L, Dacremont G, Waterham HR, Wanders RJ. Subcellular localization and physiological role of alpha-methylacyl-CoA racemase. J Lipid Res 2000;41:1890–1896.
10. Kotti TJ, Savolainen K, Helander HM, Yagi A, Novikov DK, Kalkkinen N, Conzelmann E, Hiltunen JK, Schmitz W. In mouse alpha-methylacyl-CoA racemase, the same gene product is simultaneously located in mitochondria and peroxisomes. J Biol Chem 2000;275:20887–20895.
11. Yang XJ, Wu CL, Woda BA, Dresser K, Tretiakova M, Fanger GR, Jiang Z. Expression of alphamethylacyl-CoA racemase (P504S) in atypical adenomatous hyperplasia of the prostate. Am J Surg Pathol 2002;26:921–925.
12. Jiang Z, Li C, Fischer A, Dresser K, Woda BA. Using an AMACR (P504S)/34βE12/p63 cocktail for the detection of small focal prostate carcinoma in needle biopsy. Am J Clin Pathol 2005;123:231–236.
13. Wojno KJ, Epstein JI. The utility of basal cell-specific anticytokeratin antibody (34 beta E12) in the diagnosis of prostate cancer; a review of 228 cases. Am J Surg Pathol 1995;19:251–260.
14. Signoretti S, Waltregny D, Dilks J, Isaac B, Lin D, Garraway L, Yang A, Montironi R, McKeon F, Loda M. p63 is a prostate basal cell marker and is required for prostate development. Am J Pathol 2000;157:1769–1775.
15. Weinstein M, Signoretti S, Loda M. Diagnostic utility of immunohistochemical staining for p63, a sensitive marker of prostatic basal cells. Mod Pathol 2002;15:1302–1308.
16. Parsons J, Gage W, Nelson W, De Marzo AM. p63 protein expression is rare in prostate adenocarcinoma: Implications for cancer diagnosis and carcinogenesis. Urology 2001;58:619–624.
17. Edge SB, Byrd DR, Compton CC. AJCC cancer staging manual, 7th edition. Chicago: Springer; 2010.
18. Epstein J, Allsbrook W, Amin M, Egevad LL, ISUP Grading Committee. The 2005 International Society of Urological Pathology (ISUP) Consensus conference on gleason grading of prostatic carcinoma. Am J Surg Pathol 2005;29:1228–1242.

19. Jiang Z, Woda BA, Rock KL, Xu Y, Savas L, Khan A, Pihan G, Cai F, Babcook JS, Rathanaswami P, Reed SG, Xu J, Fanger GR. P504S: A new molecular marker for the detection of prostate carcinoma. Am J Surg Pathol 2001;25(11):1397–1404.
20. Ng VW, Koh M, Tan SY, Tan PH. Is triple immunostaining with 34βE12, p63, and racemase in prostate cancer advantageous? A tissue microarray study. Am J Clin Pathol 2007; 127(2):248–253.
21. DiGiuseppe JA, Sauvageot J, Epstein JI. Increasing incidence of minimal residual cancer in radical prostatectomy specimens. Am J Surg Pathol 1997;21:174–178.
22. Epstein JI. Diagnostic criteria of limited adenocarcinoma of the prostate on needle biopsy. Hum Pathol 1995;26:223–229.
23. Epstein JI, Potter SR. The pathological interpretation and significance of prostate needle biopsy findings: Implications and current controversies. J Urol 2001;166:402–410.

3.3.1 Localization of MCT2 at peroxisomes is associated with malignant transformation in prostate cancer

Localization of MCT2 at peroxisomes is associated with malignant transformation in prostate cancer

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Conflict of interest statement

The authors declare no conflicts of interest.

Abstract

Metabolic adaptation is now considered a new hallmark of cancer, in which cancer cells exhibit high rates of glucose consumption and consequent lactate production. A previous study of MCTs expression in prostate cancer drew attention to the isoform 2 (MCT2) which was shown to be clearly expressed in prostate malignant glands, but at the cytoplasm of cancer cells, suggesting the presence of this transporter in an organelle membrane. Lately, MCT2 was suggested as a putative biomarker for prostate cancer, however, the localization and the role of MCT2 in prostate cancer cells still totally unknown.

In this work we aimed to explore more deeply MCT2 and its chaperone gp70 in prostate cancer cells using different *in vitro* models of disease progression and aggressiveness. Also, we provide a more detailed description on the localization of the other MCT isoforms at cellular level in prostate cancer cells.

We observed for the first time the presence of MCT2 in the peroxisomal membrane of prostate cancer cells and importantly, we described the interaction between MCT2 and the peroxisomal biogenesis factor 19 (PEX 19), as its chaperone, in alternative to the well-described gp70 in other tissues. We also observed that MCT2 localization in peroxisomes changes according to the disease stage. Curiously, we also observed that the distribution of MCT1 and MCT4 appeared to change across the different models of malignant transformation, suggesting alternative roles for these transporters in other cellular pathways rather than glycolysis.

All together, our results show for the first time MCT2 in prostate cancer peroxisomes interacting with PEX19, and highlight the importance of this organelle in prostate cancer disease. Also it was observed that MCT2 levels seemed to be associated with β-oxidation levels, paving the pathway for look at these transporters as targets for different metabolic pathways, different than glycolysis in cancer cells.

Introduction

Prostate cancer (PCa) is the second most common malignancy in men, which sometimes involves a challenging diagnosis [1]. Several proteins, among which alpha-methylacyl-CoA racemase (AMACR) was identified as possible new PCa biomarker [2,3].

Monocarboxylate transporters (MCTs) are proteins that facilitate the transmembrane transport of monocarboxylates, such as pyruvate and lactate [4]. In glycolytic tumors, they promote the efflux of lactic acid, being important players in the maintenance of the tumor intracellular pH, avoiding the routing to apoptosis and providing the favorable microenvironment conditions for invasion. Our recent studies point out to a consistent overexpression of MCT2 and MCT4 in prostate cancer [5,6]. Importantly, although MCT2 expression was not correlated with clinico-pathological parameters, this protein caught our attention due to its strong staining in tumour cells in contrast to a mainly negative staining in benign glands. We have also reported that, in terms of sensitivity and specificity to detect malignant glands, MCT2 staining was comparable to alpha-methylacyl-CoA racemase (AMACR), an already established biomarker in prostate cancer, raising the hypothesis that MCT2 could have an important role in prostate cancer disease [6]. However, so far, the precise localization and role of MCT2 in PCa is still unknown.

The unique biochemical feature of human MCT2 among the other MCTs is its high affinity for the transport of pyruvate, being a primary pyruvate transporter in man [7]. MCT2 is encoded by the SLC16A7 gene located in chromosome 12 (12q13) and comprises 5 coding exons. Although only one transcript is identified, there is evidence for alternatively spliced mRNA species in human and rat but no evidence of splice variants of the protein. This isoform shares approximately 50% sequence identity with MCT1, contains 478 amino acids and has a molecular weight of 52 KDa.[7]. As a higher affinity transporter, MCT2 has also a more restricted expression. Being adapted to perform the uptake of monocarboxylates into cells, MCT2 is found in tissues that use lactate as a respiratory fuel, such as brain, cardiac and skeletal muscle, kidney and liver, where lactate is the major

gluconeogenic substrate [8-13]. As MCT1, MCT2 is also found in mitochondria [12,13] and was also localized at peroxisomes in non-tumor liver fractions [14].

The presence of MCTs in mitochondria is justified by the need of a pyruvate carrier that plays a central role in carbohydrate and fat metabolism. In contrast, the presence of MCT1 and MCT2 in peroxisomes was justified as being involved in a lactate-pyruvate shuttle system present in the membrane of this organelle. This shuttle was suggested to be involved in the oxidation of NADH generated by β-oxidation, being crucial for the maintenance of peroxisomal viability and consequent β-oxidation rates [14]. This was the only study regarding this matter and it was not done in the cancer context, as so, little is known about lactate shuttles as well as the involvement of peroxisomal alterations in cancer.

In this work we aimed to unraveling MCT2 intracellular localization and expression across prostate malignant transformation using different models of disease progression in order to infer about the importance of this isoform in prostate cancer disease. Also, the other MCT isoforms where assessed in order to characterize in more detail their intracellular distribution.

Material and methods

Cell culture

In this study we have used different prostate cell lines, PNT1A (non-malignant), 22Rv1 (localized tumor), LNCaP (lymph node metastasis) and PC3 (bone metastasis). Cells were seeded in RPMI-1640 (Gibco, Invitrogen, USA) supplemented with 10% of fetal bovine serum (FBS) (PAA Laboratories GmbH, Cölbe, Germany), 1% of antibiotic (penicillin/streptomycin) (PAA Laboratories GmbH, Cölbe, Germany) and incubated at 37ºC in an atmosphere containing 5% CO_2.

Immunofluorescence and microscopy techniques

Immunofluorescence was performed in order to identify the intracellular localization of MCT1, MCT2 (sc-14926, Santa Cruz Biotechnology, Santa Cruz, CA), MCT4 and its chaperones CD147 and gp70. Fluorophores TRITC (Jackson

ImmunoResearch) and Alexa 488 (Invitrogen, Life Technologies, Grand Island, NY, USA) were used as secondary antibodies.

Immunofluorescence was performed in cells seeded on glass cover slips that were fixed after 24h with 4 % paraformaldehyde in PBS, pH 7.4 for 20 min. Afterwards cells were permeabilized with 0.2 % Triton X-100 for 10 min, blocked with 1 % BSA solution for 10 min and incubated with primary (MCT2, catalase, PEX19 and Gp70) and secondary antibodies [TRITC (Jackson ImmunoResearch) and Alexa 488 (Invitrogen, Life Technologies, Grand Island, NY, USA)] for 1 h each. Between each step, cells were extensively rinsed 3 times with PBS, pH 7.4. Lastly, cells were stained with and mounted in slides using Mowiol 4-88 containing n-propylgallate. Images were obtained using a Zeiss LSM 510 Meta Confocal setup (Carl Zeiss, Oberkochen, Germany) equipped with a plan-Apochromat 100×/1.4 oil objective.

Western Blot

Cells were lysed with specific lysis buffer (25 mM Tris-HCl, pH 8.0, 50 mM sodium chloride, 0.5% sodium deoxycholate, 0.5% Triton X-100 and a protease-inhibitor mix). To improve protein extraction, samples were passed 20 times through a 26-gauge syringe needle and incubated on a rotary mixer at for 30 min at 4ºC. After cleared by centrifugation (13300 rpm, 15 min) protein concentrations were determined by the Bradford assay (BioRad, Hercules, CA, USA). Blots were incubated with specific primary antibodies, MCT2, gp70, PEX14, Catalase, ACOX1, ACOX3 and PEX19. The antibodies were detected by a horseradish peroxidase-linked secondary antibody using an enhanced chemiluminescence system (GE Healthcare, Waukesha, WI, USA).

Immunoprecipitation

22Rv1 cells were transfected with Pex19-YFP using Turbofect *in vitro* transfection kit (Fermentas, USA), according to the manufacturer's instructions. For immunoprecipitation of Pex19-YFP we used GFP trap_M (Chromotek). Transfection with a plasmid containing GFP was used as negative control. After 48h of transfection cell pellets were incubated lysis buffer (10mM Tris-HCl, pH 7.5, 150mMNaCl, 0.5mM EDTA, 0.5%NP-40 and a protease-inhibitor mix). The lysate

was cleared by centrifugation (13300 rpm, 15 min) and diluted with dilution buffer (10mM Tris-HCl, pH 7.5, 150mM NaCl, 0.5mM EDTA and a protease-inhibitor mix). Protein concentrations were determined by the Bradford assay (BioRad, Hercules, CA, USA). Ice-cold dilution buffer was used to equilibrate beads and cell lysates were incubated for 2 h at 4°C on a rotary mixer. Beads were washed 3 times with dilution buffer and resuspended in 3x SDS-sample buffer and boiled for 10 min to elute bound proteins. Immunoprecipitated samples were separated by running in a 12.5% SDS-polyacrylamide gel.

Results

MCT2 was detected in the peroxisomes of prostate cancer cells at different levels within disease progression

The intracellular localization of MCT2 was accessed in different models of prostate cancer disease progression. As McClelland *et al* have shown a peroxisomal localization of MCT2 in non-tumor liver fractions, we firstly tested whether this protein would also be present in this organelle in prostate cancer cells. To that end, we have performed immunolocalization of MCT2 together with the peroxisomal marker catalase in different prostate cell lines: PNT1A (non-malignant), 22Rv1 (localized tumor), LNCaP (lymph node metastasis) and PC3 (bone metastasis). Our results demonstrate that, while no co-localization was observed between MCT2 and the peroxisomal marker in PNT1A, this protein is localized at peroxisomes in all the malignant cell lines (Fig 1). We have, though, observed that the localization level varied across the different models. In 22Rv1 cells, MCT2 was mainly found in the peroxisomes with a minor portion spread throughout the cytoplasm as small aggregates (Fig1 d-f). However, the ratio of peroxisomal MCT2/cytoplasm MCT2 decreased with disease progression, culminating with almost no MCT2 in the peroxisomes in PC3 cells (Fig1 j-l).

Surprisingly, the amount of MCT2 at peroxisomes appears to correlate with a change on the organelle's morphology. In fact, in cells where no MCT2 was present in peroxisomes (PNT1A), this organelle exhibits a regular phenotype (Fig 1A: a-c). Curiously, in 22Rv1 cells (where MCT2 was mainly observed in

peroxisomes) this organelle appears somewhat in clusters (Fig. 1A: d-f) LNCaP cells exhibit more distributed rounder peroxisomes with only a few clusters (Fig.1A: g-i). In PC3, the highly metastatic model, peroxisomes appear similar to the ones of the non-malignant cells PNT1A (Fig 1A: j-l). Strikingly, the expression level of MCT2 increases from non-tumor (PNT1A) to localized malignant cells (22Rv1), correlating to its change in localization from cytoplasmic to peroxisomal (Fig 1B). This increase in expression was also observed for Pex14 (a peroxisomal membrane protein) (Fig 1C), suggesting an increase in peroxisomal membrane surface/number that accompanies the malignant transformation.

MCT2 travels to the peroxisomal membranes trough PEX19

The quick movement of monocarboxylates across the membranes is imperative for cellular metabolism. These proteins are thought to require chaperones such as CD147 in the case of MCT1 and MCT4, or gp70 in the case of MCT2, for appropriate expression and activity in the plasma membrane [15]. Since MCT2 was the only isoform found to localize in peroxisomes in prostate cancer cells, we aimed at unraveling its targeting mechanism to this organelle. A previous study was unable to find gp70 expression in human prostate samples that exhibited MCT2 expression [5]. However, our studies with confocal microscopy allowed us to observe some, although scarce, gp70 distributed in the cytoplasm without any co-localization with peroxisomal markers (Fig 2 a-l). Hence, the protein that behaves as chaperone for MCT2 transport to peroxisomes in prostate cells is still unknown.

As Pex19 is the main protein responsible for the transport of peroxisomal membrane proteins to this organelle, we tested whether this protein could act as a chaperone for MCT2 in these cells. Being 22RV1 the cell line where MCT2 was mainly present at peroxisomes, it was the chosen model to study the possible interaction between MCT2 and PEX19. Immunoprecipitation experiments have shown, indeed, an interaction between MCT2 and PEX19 (Fig 3). These results suggest a highjack of the peroxisomal transport mechanism by the prostate cancer cells.

The presence of MCT2 at peroxisomes seems to be related with an increase in the rates of peroxisomal β-oxidation

McClelland *et al* observed a decrease in β-oxidation upon MCTs inhibition, suggesting that the presence of MCT2 at peroxisomes of non-malignant liver cells would be essential for the maintenance of peroxisomal viability and consequently β-oxidation rates. Hence, we hypothesized that the increase of MCT2 expression from non-malignant prostate cells (PNT1A) to localized prostate tumor cells (22RV1) could be related with an increase in peroxisomal β-oxidation. In fact, we observed an increase in the expression levels of ACOX1 and ACOX-3, central proteins in the β-oxidation pathway (Fig 4). These results interestingly suggest that, indeed, the increase in MCT2 expression levels as well as its presence at peroxisomes, are related to an increase in β-oxidation levels which may be crucial for malignant transformation.

The distribution of other MCTs isoforms and CD147 at intracellular level suggests an additional role for these proteins in prostate cancer

MCT1, MCT4 and CD147 expressions were also evaluated in this study. MCT1 showed a strong nuclear expression in PNT1a and 22RV1, decreasing for LNCaP and PC3, where this transporter was mainly found in the cytoplasm and plasma membrane (Fig.5). MCT4 however was shown to be present in the cytoplasm of the less tumorigenic cell lines 22RV1 and LNCaP in contrast to the highly metastatic PC3 where it was clearly present at the plasma membrane (Fig.6). CD147 was present in the plasma membrane, cytoplasm and nuclear envelope (Fig.7).

Interestingly, some co-localization between MCTs 1 and 4 and peroxisomes, was also verified, however it was less evident than for MCT2.

Discussion

It is now widely accepted that the transport of lactate and pyruvate is mediated by a family of H+/monocarboxylate transporters (MCTs) known for their specificity to transport short chain monocarboxylates. They form an integral part of proposed

shuttles that transport lactate between cells (cell–cell lactate shuttle) and within cells (intracellular lactate shuttle).

A previous study of MCTs expression in prostate cancer drew attention to MCT2 that was shown to be clearly expressed in prostate malignant glands but at the cytoplasm, suggesting the involvement of this transporter in an organelle membrane [6], however there were no studies describing the existence of MCT2 in the organelles of prostate cancer cells.

It is known, that once in the mitochondrial matrix, lactate and pyruvate undergo further breakdown making it difficult to infer their respective roles in the shuttling of reducing equivalents to the electron transport chain. Peroxisomes, on the other hand, lack the enzymes to catabolize monocarboxylates and although they are known to contain LDH [16], they are not known to contain any other glycolytic or any tricarboxylic acid cycle enzymes. One of the many functions of liver peroxisomes is the β-oxidation of long branched-chain fatty acids and for the maintenance of these reactions inside the peroxisome, a redox shuttle system exist across the peroxisomal membrane to reoxidize NADH. It is essential for the continuous functioning of peroxisomes that proper intra-organelle reduction–oxidation (redox) balance is maintained that ensures the reoxidation of NADH produced by β-oxidation and the continuous breakdown of fatty acids. Redox balance in many organelles is maintained in part by substrate shuttles. This would entail a shuttle system in peroxisomes that neither generates products nor consumes any substrates.

McClelland *et al* proposed that a redox shuttle system that consists of a substrate cycle between lactate and pyruvate, as so, to play their role in peroxisomal redox balance, pyruvate and lactate must be transported across the peroxisomal membrane [14].

In the present study we describe for the first time that MCT2 is present in human prostate peroxisomes and a possible explanation for how these MCT2 ends up in the peroxisomal membrane was given by the evident interaction of MCT2 with PEX19 which might act as chaperone. The absence of glycolysis in this organelle strongly suggests that this shuttle serves to maintain redox balance and not glycolysis or some other metabolic function. Consequently, we point out the importance of peroxisomes in prostate cancer cells that as it was observed a clear

change their morphology across prostate malignant transformation, which provides further evidence for the dynamic nature of the peroxisomes across disease progression. Importantly, the frequency of peroxisomal clusters and the co-localization with MCT2 that was mainly observed in the transition from the benign model (PNT1a) to the malignant model (22RV1), point to the involvement of these alterations in tumour initiation. Finally, the possibility of having MCT1 and MCT4 in the peroxisomal membrane, however less evident than for MCT2, corroborates the hypothesis of alternative roles for these transporters in metabolic pathways different from glycolysis in the present cells. However, further studies are needed to understand the role of these MCT isoforms in prostate peroxisomes.

References

1. Comperat, E., J. Varinot, and J.R. Srigley, *[Benign mimickers of the prostate cancer. Diagnostic challenges].* Ann Pathol, 2013. **33**(4): p. 237-46.
2. Schmitz, W., R. Fingerhut, and E. Conzelmann, *Purification and properties of an alpha-methylacyl-CoA racemase from rat liver.* Eur J Biochem, 1994. **222**(2): p. 313-23.
3. Lloyd, M.D., et al., *Alpha-methylacyl-CoA racemase--an 'obscure' metabolic enzyme takes centre stage.* FEBS J, 2008. **275**(6): p. 1089-102.
4. Halestrap, A.P., *The SLC16 gene family - structure, role and regulation in health and disease.* Mol Aspects Med, 2013. **34**(2-3): p. 337-49.
5. Pertega-Gomes, N., et al., *Monocarboxylate transporter 4 (MCT4) and CD147 overexpression is associated with poor prognosis in prostate cancer.* BMC Cancer, 2011. **11**: p. 312.
6. Pertega-Gomes, N., et al., *Monocarboxylate transporter 2 (MCT2) as putative biomarker in prostate cancer.* Prostate, 2013. **73**(7): p. 763-9.
7. Lin, R.Y., et al., *Human monocarboxylate transporter 2 (MCT2) is a high affinity pyruvate transporter.* J Biol Chem, 1998. **273**(44): p. 28959-65.
8. Pierre, K., et al., *Cell-specific localization of monocarboxylate transporters, MCT1 and MCT2, in the adult mouse brain revealed by double immunohistochemical labeling and confocal microscopy.* Neuroscience, 2000. **100**(3): p. 617-27.
9. Pierre, K., P.J. Magistretti, and L. Pellerin, *MCT2 is a major neuronal monocarboxylate transporter in the adult mouse brain.* J Cereb Blood Flow Metab, 2002. **22**(5): p. 586-95.
10. Debernardi, R., et al., *Cell-specific expression pattern of monocarboxylate transporters in astrocytes and neurons observed in different mouse brain cortical cell cultures.* J Neurosci Res, 2003. **73**(2): p. 141-55.
11. Pellerin, L., et al., *Cellular and subcellular distribution of monocarboxylate transporters in cultured brain cells and in the adult brain.* J Neurosci Res, 2005. **79**(1-2): p. 55-64.

12. Yoshida, Y., et al., *Negligible direct lactate oxidation in subsarcolemmal and intermyofibrillar mitochondria obtained from red and white rat skeletal muscle.* J Physiol, 2007. **582**(Pt 3): p. 1317-35.
13. Benton, C.R., et al., *Monocarboxylate transporters in subsarcolemmal and intermyofibrillar mitochondria.* Biochem Biophys Res Commun, 2004. **323**(1): p. 249-53.
14. McClelland, G.B., et al., *Peroxisomal membrane monocarboxylate transporters: evidence for a redox shuttle system?* Biochem Biophys Res Commun, 2003. **304**(1): p. 130-5.
15. Wilson, M.C., et al., *Basigin (CD147) is the target for organomercurial inhibition of monocarboxylate transporter isoforms 1 and 4: the ancillary protein for the insensitive MCT2 is EMBIGIN (gp70).* J Biol Chem, 2005. **280**(29): p. 27213-21.
16. Baumgart, E., et al., *L-lactate dehydrogenase A4- and A3B isoforms are bona fide peroxisomal enzymes in rat liver. Evidence for involvement in intraperoxisomal NADH reoxidation.* J Biol Chem, 1996. **271**(7): p. 3846-55.

Figures

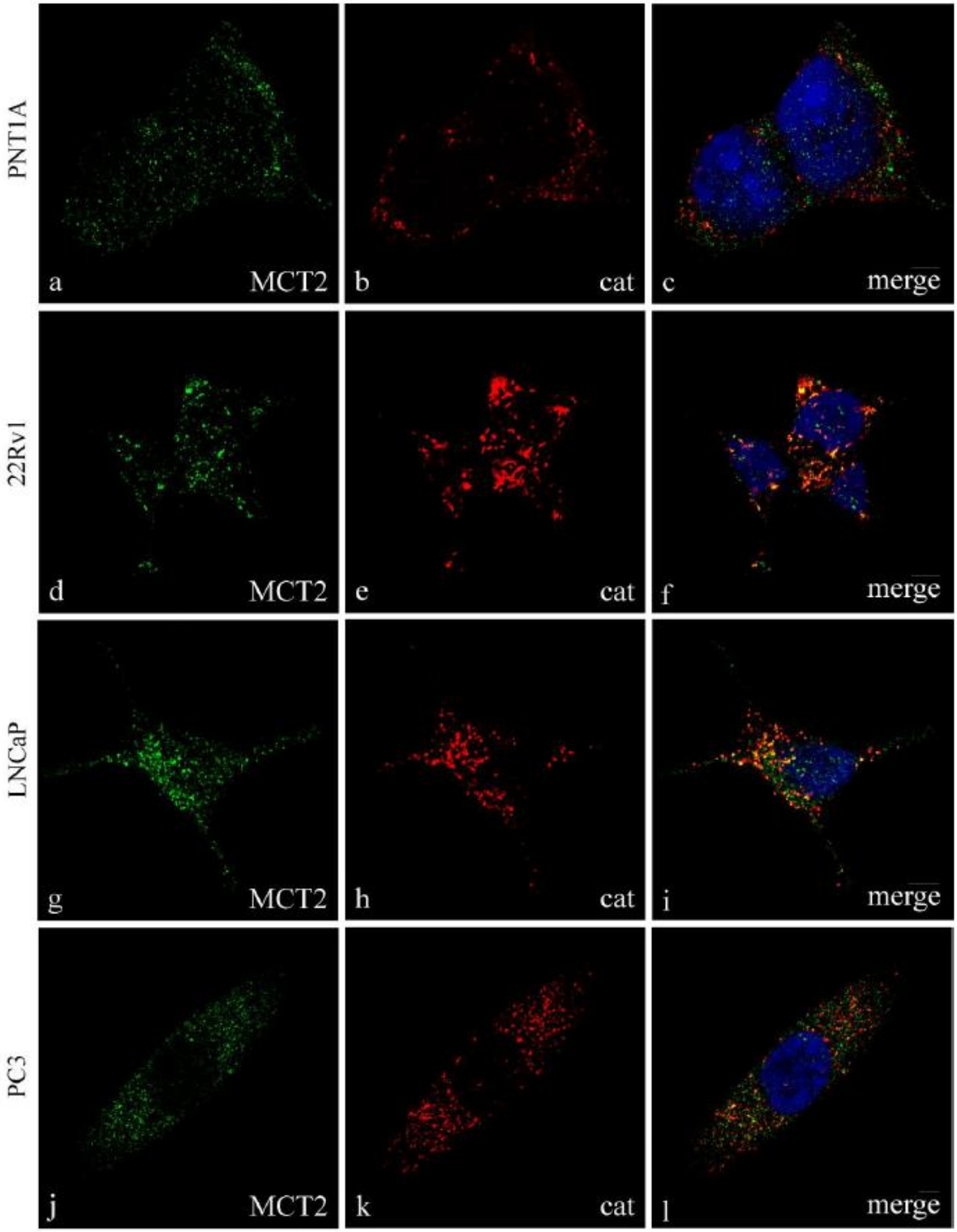


Figure 1 A) Immunohistochemical images of different human prostate cell lines PNT1a (Figure 1a-c), 22RV1(Figure 1d-f), LNCaP (Figure 1g-i) and PC3 (Figure 1j-l). Superposition of signals from probes for MCT2 (green), and Catalase (red) shows clear colocalization (yellow in the peroxisomes of prostate cancer cells).

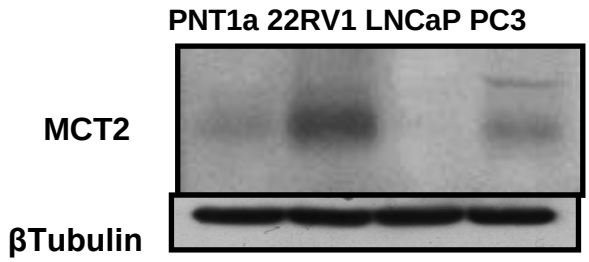


Figure 1 B) Western blot analysis showing the levels of MCT2 in the different prostate cell line models.

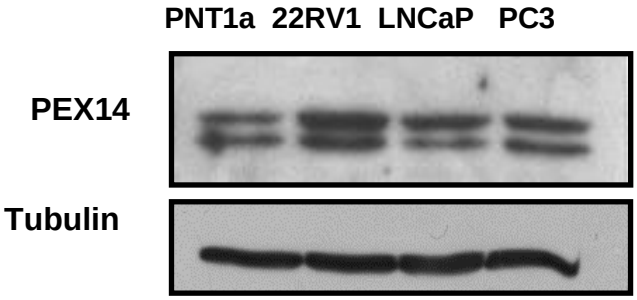


Figure 1 C) Western blot analysis, showing the expression levels of the peroxisomal protein PEX-14 in the different in vitro models of prostate cancer progression (PNT1a, 22RV1, LNCaP and PC3).

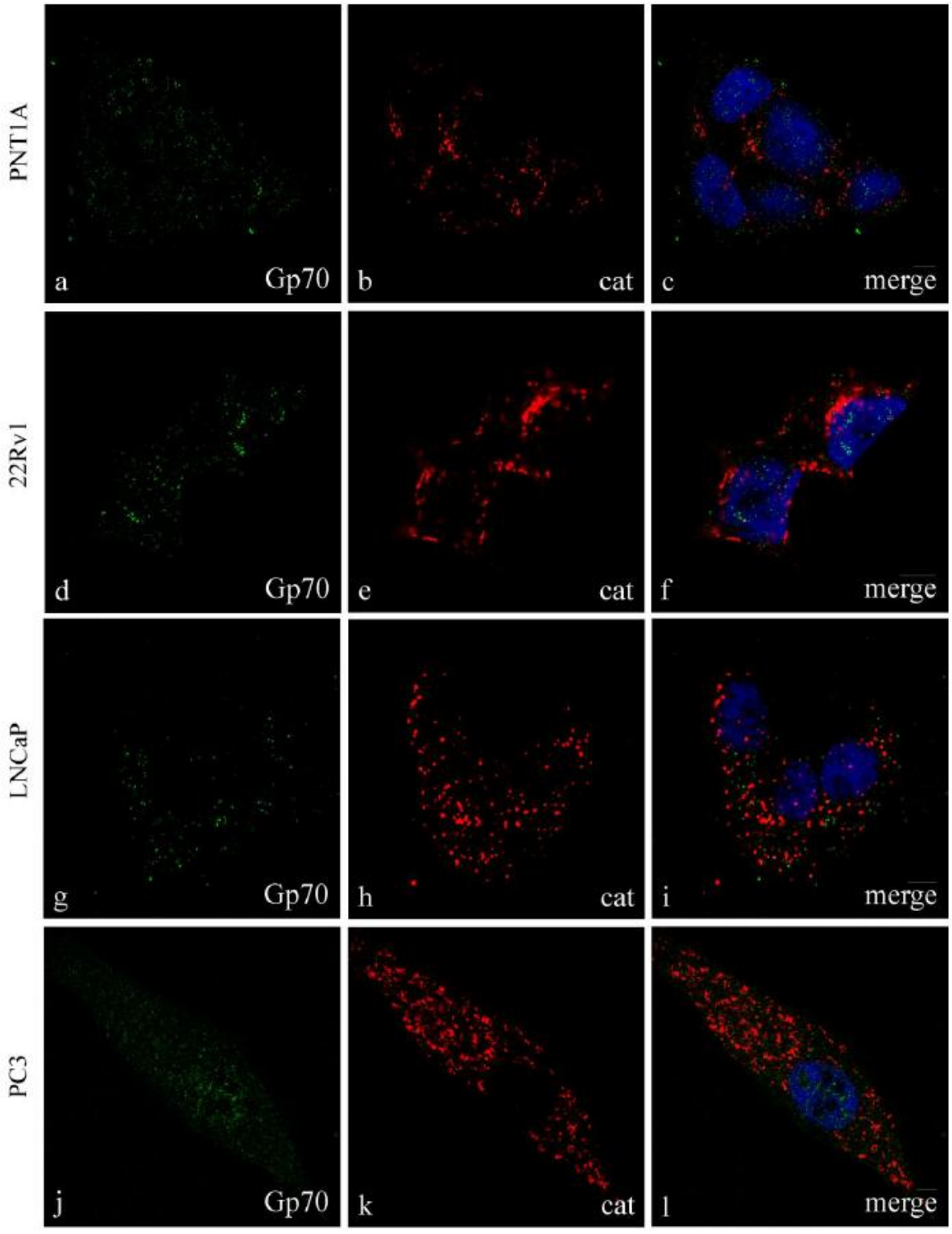


Figure 2. Immunohistochemical images of different human prostate cell lines PNT1a (Figure 2a-c), 22RV1 (Figure 2D-F), LNCaP (Figure 2G-I) and PC3 (Figure 2J-L). Superposition of signals from probes for gp70 (green), and Catalase (red) showed clear colocalization (yellow in the peroxisomes of prostate cancer cells).

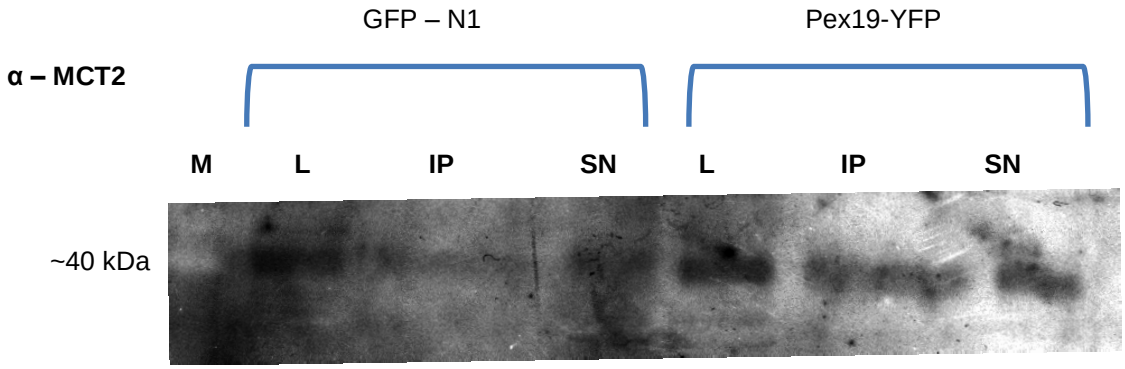
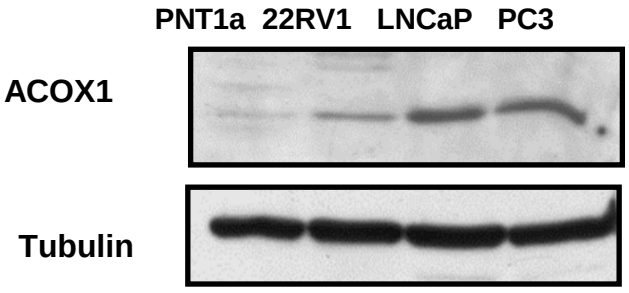


Figure 3. Interaction of MCT2 with PEX19 using co-immunoprecipitation (Co-IP) and Western blot in 22RV1 cell line.

A)



B)

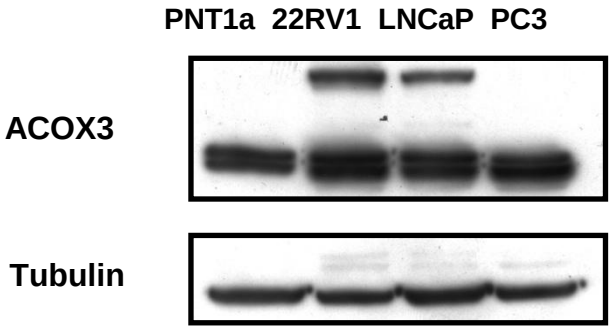


Figure 4. Western blot analysis, showing the expression levels of the proteins ACOX-1 (A) and ACOX-3 (B) involved in the peroxisomal branched chain fatty acid, in the different *in vitro* models of prostate cancer progression (PNT1a, 22RV1, LNCaP and PC3).

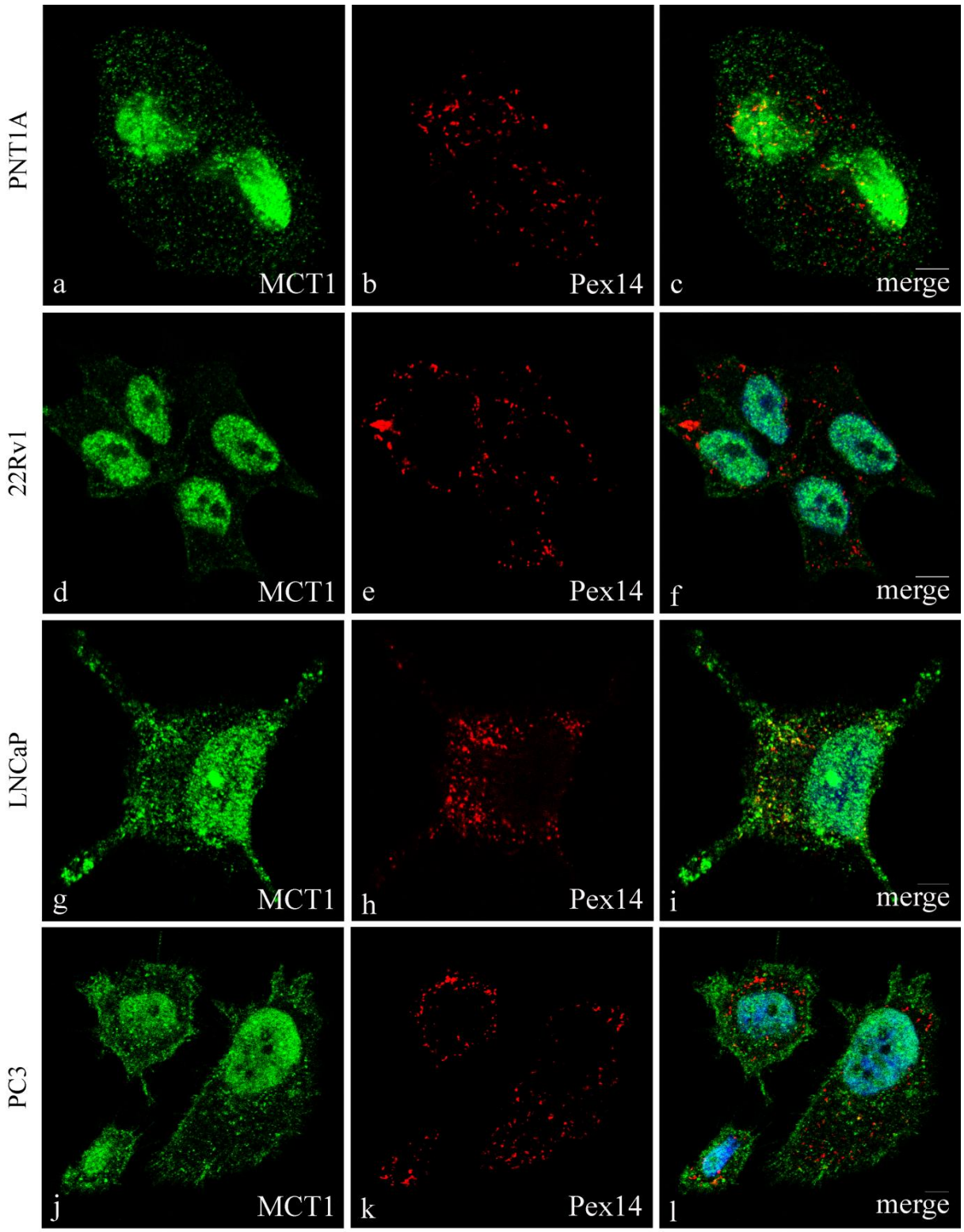


Figure 5. Immunohistochemical images of different human prostate cell lines PNT1a (Figure 7A) ,22RV1(Figure 7B), LNCaP (Figure 7C) and PC3 (Figure 7D) showing probes for MCT1 (green). A clear presence of MCT1 in both plasma membrane and nucleus was observed in all the models studied.

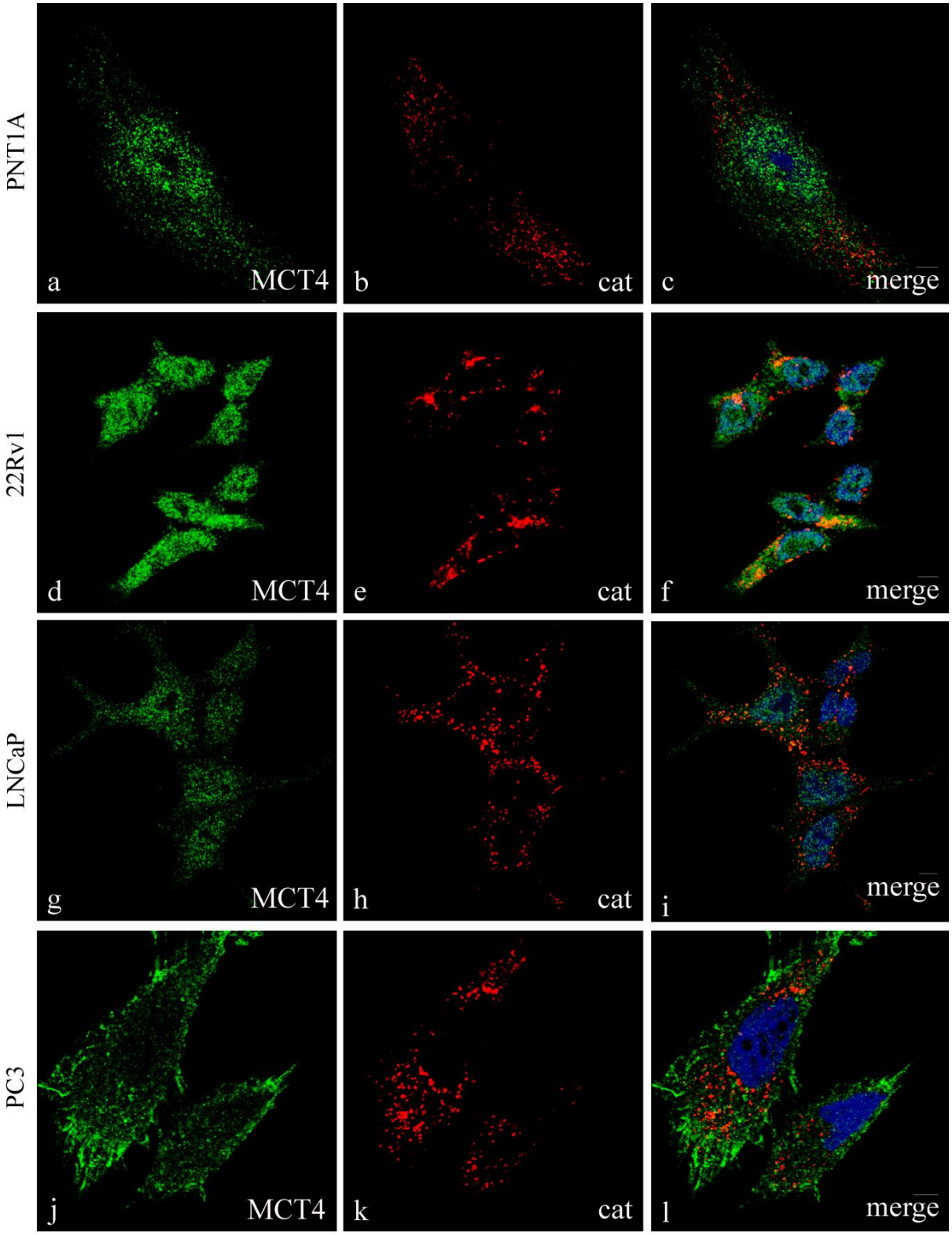


Figure 6. Immunohistochemical images of different human prostate cell lines PNT1a (A-C), 22RV1(D-F), LNCaP (G-I) and PC3 (J-L). Superposition of signals from probes for MCT4 (green), and Catalase (red) showed clear colocalization (D, yellow in the peroxisomes of prostate cancer cells).

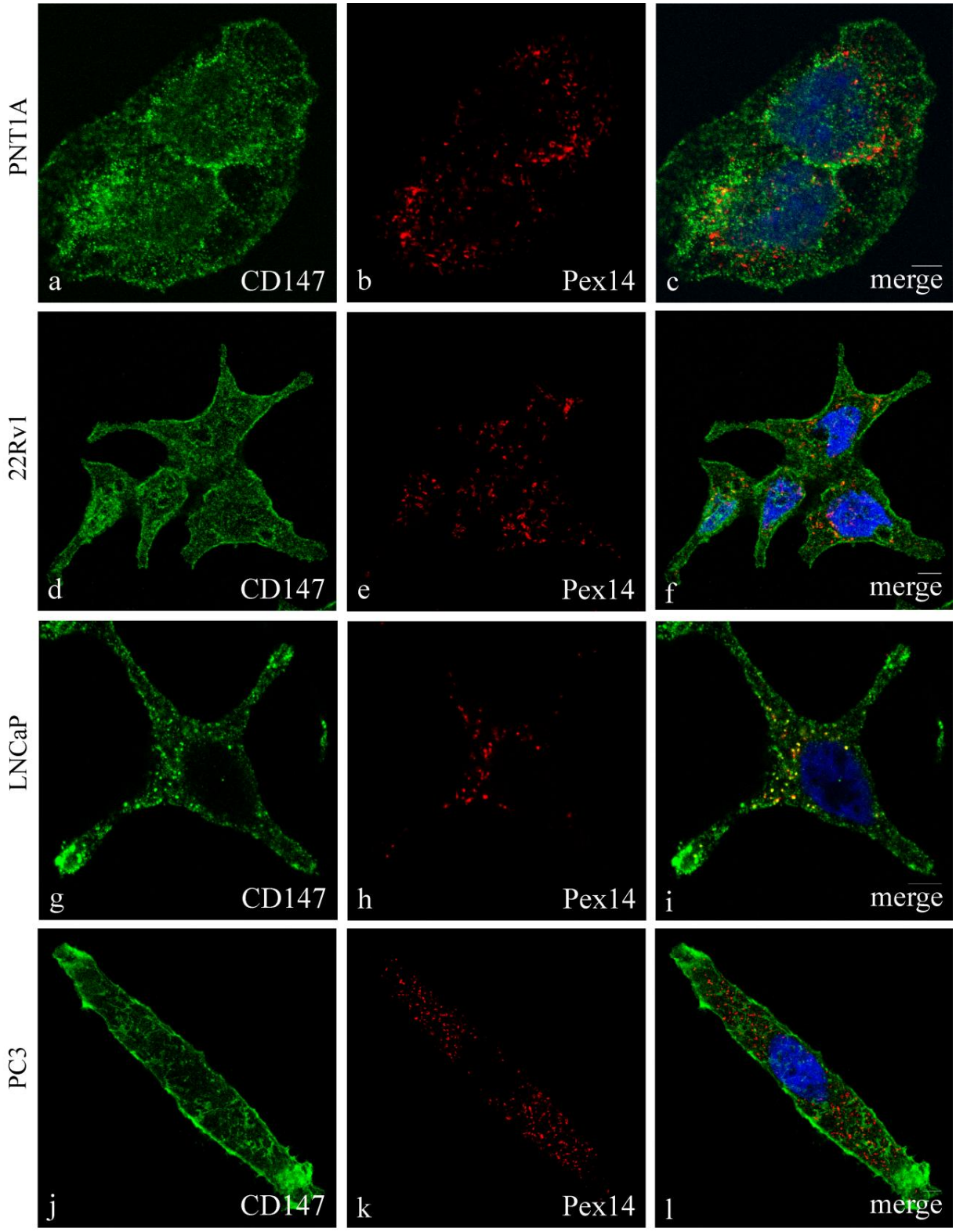


Figure 7. Immunohistochemical images of different human prostate cell lines PNT1a (a-c), 22RV1(d-f), LNCaP (g-i) and PC3 (j-l) showing probes for CD147 (green). A clear presence of CD147 in both plasma membrane and perinuclear envelope was observed in all the models studied.

CHAPTER 4. EXPLOTATION OF METABOLIC ALTERATIONS AS TARGETS FOR THERAPY

4.1 CHAPTER OVERVIEW

We firstly described overexpression of MCT2 and MCT4 in PCa tissues when compared to the normal counterparts, however, the role of MCTs in PCa metabolism is still largely unknown. The presence of MCT4 in the cytoplasm of prostate cancer cells instead of plasma membrane together with the expression of MCT1 in both non-malignant and malignant glands, suggested that prostate cells may not firstly rely on glycolytic metabolism as the majority of tumours. This verification turned this work even more challenging in an attempt to contextualize the role of different MCTs isoforms across prostate malignant transformation and progression.

As stated before, it remains unclear what is the primary energetic pathway in PCa and therefore which metabolic pathway represents the most appropriate target for metabolic inhibition in PCa. There are studies reporting the presence of hypoxia in PCa, pointing to the presence of a glycolytic phenotype, however other studies report that PCa is characterized by low rates of glycolysis and a low FDG activity on PET imaging. This fact raises the hypothesis that if glucose consumption is not elevated in PCa, alternative metabolic pathways must provide the energy needed for cancer cell proliferation and growth. Some evidence support a crucial role of fatty acid-related metabolism in the pathogenesis and progression of prostate malignancy. However, the reports are scarce with low number of clinical samples and with no information regarding the clinico-pathological significance of these alterations; as so, PCa metabolism is still largely unknown.

This chapter includes results already submitted for publication, showing the expression pattern of several metabolic-related proteins involved in glycolytic and fatty acid oxidation pathways from benign prostate to metastatic PCa, investigating the prognostic impact of their expression. Also, using different *in vitro* models of disease progression we tested how the inhibition of these pathways affect the different models of prostate cancer cells in order to establish a rationale for metabolic phenotypes across prostate malignant transformation and progression.

Overall, the results presented here indicate a switch from less aggressive tumor to glycolytic metabolism in the highly aggressive and metastatic prostate tumor

and underscores the plasticity of bioenergetic pathways in prostate cancer cells which might have crucial implications at prognostic and therapeutic level.

4.2 PUBLISHED RESULTS

The results presented in this chapter were:

(i) Submitted for publication as an original article in an international peer reviewed journal

Pértega-Gomes N, Vizcaíno JR, Sousa S, Coelho R, Attig J, Jurmeister S, Oliveira E, Pereira L, Pinheiro C, Jerónimo C, Henrique RM, Lobo da Cunha A, Lopes C, Maximo V and Baltazar F "Metabolic heterogeneity in prostate cancer is linked to disease progression and aggressiveness". 2013.

(ii) Invited for publication as an original article in an international peer reviewed journal

Pértega-Gomes N. and Baltazar F. "MCTs as targets for prostate cancer therapy?" invited work to be published in **Advances in Prostate Cancer Research and Treatment** journal. 2014.

(iii) Presented as oral communication in the following international scientific meeting

***Pértega-Gomes N**, Vizcaíno JR, Lopes C and Baltazar F. "Tracing the metabolic profile of prostate cancer development" work presented at 23rd European Congress of Pathology at Helsinki, Finland. 2011.*

***Pértega-Gomes N.,** Vizcaíno JR, Lopes C and Baltazar F. "Exploitation of metabolic alterations as importante prognostic, diagnostic and therapeutic tools in prostate cancer". Work presented at 20th Meeting of the EAU Section of Urological Research (ESUR). 25-27 October 2012, Strasbourg, France. 2012.*

(iv) Presented as oral communication in the following national scientific meeting:

Pértega-Gomes N, Lourenço T, Vizcaíno JR, Lopes C and Baltazar F. "Study of Metabolic profile of localized and metastatic tumour of the prostate. Monocarboxylate transporters as therapeutic targets" work presented at XVI Workshop on Oncological Urology, Carvoeiro, Portugal. 2011.

Pértega-Gomes N, Lourenço T, Vizcaíno JR, Lopes C and Baltazar F. et al. "A metabolic switch could be involved in prostate cancer aggressiveness."Work presented at the 12º Congress of the Portuguese Society of Oncology, Algarve, Portugal. 2011.

(v) Presented as <u>poster in the following international scientific meeting:</u>

Pértega-Gomes N, Lourenço T, Miranda-Gonçalves V, Vízcaíno JR, Oliveira E, Jerónimo C, Lopes C, Lobo da Cunha A. and Baltazar F. "Emerging roles for Monocarboxylate Transporters in prostate cancer metabolism". at 2nd Symposium of the International Society for Proton Dynamics in Cancer in Nice, France. 2011.

Miranda-Gonçalves V, **Pértega-Gomes N,** Lourenço T, Pinheiro C, Jerónimo C, Vizcaíno JR, Lopes C, and Baltazar F. "Prostate Cancer: exploitation of monocarboxylate transporters as potential therapeutic targets". at the 39th Meeting of International Society of Oncology and Biomarkers in Florence, Italy. 2011.

Pértega-Gomes N., Vizcaíno JR, Lourenço T, Lopes C. and Baltazar F "Exploiting metabolic alterations in prostate cancer diagnosis and prognosis" at XXI Porto Cancer Meeting, Porto. 2012.

Pértega-Gomes N, Vizcaíno JR, Lourenço T, Lopes C. and Baltazar F "Tracing the metabolic profile of prostate cancer progression. Roles for monocarboxylate transporters (MCTs) in prostate cancer metabolism" at EACR Annual Congress, Barcelona. 2012.

Pértega-Gomes N, Vizcaíno JR, Lourenço T, Lopes C. and Baltazar F. "Clinical impact of the metabolic phenotype of prostate cancer: Role of monocarboxylate transportes (MCTs)." at São Paulo Advanced School of Comparative Oncology. September 30th to October 6th. Águas de São Pedro-São Paulo, Brasil. 2012.

Pértega-Gomes N, Vizcaíno JR, Lourenço T, Lopes C. and Baltazar F. "Metabolic phenotypes associated with distinct androgen-responsive conditions can be a valuable tool for diagnostic and therapeutic options in prostate cancer." at A one day symposium with Carlos Caldas sponsored by EACR. 29TH October, Porto, Portugal. 2012.

(vi) <u>Were recognized with the the following prizes/awards:</u>

Pértega-Gomes N, Vizcaíno JR, Lopes C and Baltazar F **"**Tracing the metabolic profile of prostate cancer development**". Bursary Award** given by the European Society of Pathology (ESP) to attend 23º Congress of the European Society of Pathology meeting in Helsinki, Finland.

Pértega-Gomes N, Lourenço T., Vizcaíno JR, Lopes C and Baltazar F. "Study of Metabolic profile of localized and metastatic tumour of the prostate. Monocarboxylate transporters as therapeutic targets" **1st Prize** (Janssen Award) and **Honorable Mention** given by for **best poster** and **scientific communication** in National Congress of Urology.

Pértega-Gomes N, Lourenço T., Vizcaíno JR, Lopes C and Baltazar F. 2011. "A metabolic switch could be involved in prostate cancer aggressiveness. **1st Prize** for **best poster** and **scientific communication** in 12º National Congress of Oncology.

Pértega-Gomes N, Vizcaíno JR, Lourenço T, Lopes C. and Baltazar F. 2012. "Clinical impact of the metabolic phenotype of prostate cancer: Role of monocarboxylate transportes (MCTs)." **Bursary award** given by FAPESP - São Paulo Research Foundation to attend ESPCA-School of Comparative Oncology at Águas de São Pedro-São Paulo, Brasil, 2012.

4.3.1 Metabolic heterogeneity in prostate cancer is linked to disease progression and aggressiveness

Metabolic heterogeneity in prostate cancer is linked to disease progression and aggressiveness

Running Title: Metabolic heterogeneity in PCa progression

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Conflict of interest statement

The authors declare no conflicts of interest.

Abstract

Metabolic adaptation is now considered a new hallmark of cancer, in which cancer cells exhibit high rates of glucose consumption with consequent lactate production. However, the dominant energetic pathway as well as the clinical impact of altered cellular metabolism in prostate cancer is still largely unknown.
In an attempt to understand the metabolic alterations during prostate cancer progression and how these alterations could represent suitable tools for prostate cancer at diagnostic, prognostic and therapeutic level, we studied the expression of several key metabolic-related proteins in 480 human prostate samples and using *in vitro* models we assessed metabolic changes associated with malignant transformation/progression and how their manipulation affects different models of prostate cancer cells. Also we provided an insight into the cell mitochondrial features and used publicly available genome-wide expression profiling datasets to assess the expression of the genes that codify metabolic-related proteins during prostate cancer progression.

This study provides evidence for increased glycolytic phenotype mainly in the advanced stages of prostate cancer, and its correlation with poor prognosis whereas the localized tumour showed more consistently upregulation of proteins involved in peroxisomal branched-chain fatty acid oxidation without any association with clinic-pathological data. Importantly, experiments using *in vitro* models showed that cell lines derived from distant metastasis are more glycolytic, sensitive to glycolytic inhibitors and with lower energy efficiency at the mitochondrial level.

This is the most comprehensive study reporting the metabolic differences across prostate malignant transformation and correlating the metabolic heterogeneity of prostate cancer cells with unfavorable prognosis, showing that metabolic changes have a strong potential to be explored as diagnostic, prognostic and therapeutic tools in prostate cancer.

Keywords: prostate cancer, cancer metabolism, monocarboxylate transporters, glycolytic metabolism, fatty acid oxidation, metabolic switch.

Introduction

Metabolic changes during malignant transformation have been noted for many years. Warburg firstly reported that cancer cells preferentially rely on glycolysis for energy production, even in the presence of oxygen, leading to the production of high levels of lactate [1]. Our group described for the first time the expression and the involvement of monocarboxylate transporters (MCTs), proteins which facilitate the transmembrane transport of lactate, as promising targets in cancer therapy in several human cancers [2-7]. Regarding prostate cancer, although we have found some important correlations with prognostic parameters, the presence of MCT1 in both tumour cells and non-malignant tissues as well as the absence of MCT4 at the plasma membrane of cancer cells lead us to hypothesize that prostate cancer might rely less than most tumours on aerobic glycolysis [8-9]. However, it remains unclear what is the primary energetic pathway in PCa and therefore which metabolic pathway represents the most appropriate target for metabolic inhibition in PCa. There are studies reporting the presence of hypoxia in PCa, pointing to the presence of a glycolytic phenotype [10-13], however, other studies report that PCa is characterized by low rates of glycolysis and a low 2-fluoro-2-deoxy-D-glucose FDG activity on positron emission tomography PET imaging [14-16]. This fact raises the hypothesis that if glucose consumption is not elevated in PCa, alternative metabolic pathways must provide the energy needed for cancer cell proliferation and growth. Some evidence supports a crucial role of fatty acid-related metabolism in the pathogenesis and progression of prostate malignancy [17-18]. However, the reports are scarce with low number of clinical samples and with no information regarding the clinico-pathological significance of these alterations, thus, PCa metabolism is still largely unknown.

Making use of a large human casuistic and different in vitro models of prostate cancer disease progression, this study aims to address for the first time the metabolic heterogeneity across prostate malignant transformation and progression and infer about its significance in the clinical context.

Materials and methods

Patients' samples

Prostate tissues were obtained from 480 patients with a median age of 64 years old, who performed radical prostatectomy between 1993 and 2010. Samples, including 203 non-neoplastic, 176 high-grade prostatic intraepithelial neoplasia (PIN) and 480 neoplastic tissues, and clinico-pathological data were retrieved from the files of the Department of Pathology, Centro Hospitalar do Porto and organized into tissue microarray blocks (TMAs), as previously described [9]. Benign samples were obtained from 12 patients undergoing radical cystoprostatectomy for transitional cell carcinoma of the bladder and 8 metastatic PCa cases were obtained from clinical biopsy samples. The present study was previously approved by Local Ethical Review Committees.

Cell lines and cell culture

Four human prostate cell lines representing different relevant features of prostatic adenocarcinoma were used. The selected cells lines were 22RV1, LNCaP, PC3 and DU145 (ATCC-American Type Culture Collection, MD, USA). Cell lines were grown in RPMI-1640 cell culture medium supplemented with 10% Fetal Bovine Serum (FBS) (GIBCO®, Invitrogen,Carlsbad, CA) and 1% Penicillin-Streptomycin (P-S) (GIBCO®, Invitrogen, Carlsbad, CA).

Immunohistochemistry (IHC)

The expression of lactate transporters (MCT1, 2 and 4), glucose transporters (GLUT-1 and GLUT-12), proteins involved in the glycolytic cascade (HKII, LDHV and PDKI), cellular biomarkers of hypoxia (CAIX and HIF-1alpha) and finally proteins involved in fatty acid oxidation (AMACR, ACOX-3 and DBP) were analyzed in prostate tissue samples using a combined score system previously described [2-7]. IHC technique was performed according to the detailed information given in **Table 1** and IHC evaluation was performed blindly by two independent observers that assessed the intensity and the extention of the staining as previously described [9].

Heat map construction

A heat map representing the staining of each protein for each clinical case was constructed using R statistical software for *statistical* computing and graphics.

Western blot

Western blot analysis was performed for protein expression in all cell lines. Details are given in **Table 2**.

Drugs

Thioridazine (TZ), phytanic acid (PA) and oxamic acid (OA) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) to prepare the stock solutions from which the working solutions were made.

Metabolism assay (extracellular glucose and lactate measurements)

Cells were plated in 24-well plates until confluence. A concentration of 40mM of OA (according to the concentration range used in the literature) was used. Glucose consumption and lactate production were quantified before and after treatment using commercial kits (Roche and Spinreact, respectively), as previously described [2].

Cell viability assay

The effect of treatment with TZ on cell biomass was determined at 24, 48 and 72 hours by MTS assay (Sigma-Aldrich), according to the manufacturer's recommendations. IC_{50} values (i.e., TZ concentration that corresponds to 50% of cell growth inhibition) were estimated from 3 independent experiments. All values were normalized for untreated controls. MTS assay was also used to assess the effects of increasing concentrations of phytanic acid in cell lines.

Ultrastructural studies

Fresh human tissues were harvested from patients undergoing radical prostatectomy at Portuguese Institute of Oncology, Porto. Prostate tissue was reseated from peripheral zone and immediately fixed following surgical removal. LNCaP and PC3 cells were also used for electron microscopy analysis. Samples were processed and observed using a *transmission electron microscope* as previously described [19].

Mitochondrial DNA (mtDNA) characterization, haplogroup affiliation and cell respiratory efficiency

The complete mtDNA of the cell lines LNCaP and PC3 was amplified in 32 overlapping fragment with primers and PCR conditions (described in Table S1, Supplementary data). The same primers were used to directly sequence both strands of the fragments using the Promegafmol® DNA Cycle sequencing System and the Usb Thermo Sequence Radiolabelled Terminator Cycle Sequencing Kits. Sequences were aligned and compared with the revised Cambridge Reference Sequence [20] , by using the software BioEdit. The diversity observed in each cell line was automatically classified by using the software mtDNA-GeneSyn [21], and a pathogenicity score [22] was inferred for the non-synonymous polymorphisms based upon the list provided in Pereira et al. [23] for all possible non-synonymous mutations which can occur in the human mtDNA by point mutations. Haplotypes were also assigned to haplogroups by using the software Haplogrep [24], which also indicates the polymorphisms that are potentially private and the ones shared by ancestry.

Analysis of cell respiratory efficiency

The activity of the mitochondrial complex I (NADH:ubiquinone oxidoreductase) was evaluated enzymatically by using the Complex I Enzyme Activity Microplate Assay Kit (ab109721) (Abcam®), in cells cultures with 70-80% confluence, according to the manufacturer's instructions. The global cellular ATP levels

were quantified using Luminescent ATP Detection Assay Kit (ab113849, Abcam).

Public genome-wide expression profiling datasets analysis

The Varambally dataset (Varambally *et al.,* 2005) containing the expression profiling data from 16 microdisected samples representing benign prostate, primary tumour and metastases, was used. Also, the Glinsky dataset (Glinsky *et al.*, 2004), which contains expression data and clinical follow-up for 79 prostate cancer patients, was used to determine if the expression of any of the proteins studied correlate with clinical outcome.

Statistical analysis

Data from human tissue samples were analyzed with SPSS statistical software (version 18.0; SPSS) using the Pearson's χ^2 test, with the threshold for significance being $p \leq 0.05$. For the in vitro studies, the GraphPad prism 5 software was used, with the Student's t-test, considering significant values to be $p \leq 0.05$.

Results

The expression profile of key metabolic-related proteins changes across prostate malignant transformation

Figure 1 shows the final staining score presented by each protein studied in each case of benign prostate tissue (BT), non-neoplastic prostate tissue (NT), PIN lesions (PIN), tumour tissue (TT) and metastatic tumour tissue (MT). The colours distinguish the cases in which the final score was negative (0), weak (1), moderate (2) and strong (3). We observed important changes in the expression of all the metabolic-related proteins studied from benign and/or adjacent non-neoplastic prostate tissue to PIN lesions and to primary tumour. Firstly, an increase in MCT2, MCT4, GLUT-1, GLUT-12, LDHV, PDK1, CAIX, AMACR, ACOX-3 and DBP expressions was observed from BT and NT to TT.

Interestingly, an evident increase was verified from BT or NN to PIN and TT for proteins involved in fatty acid oxidation whereas proteins involved in the glycolytic pathway, namely MCT4, LDHV, HKII, PDKI and CAIX were positive in all metastatic samples. The specific percentage of positive cases for each protein in different tissues, as well as, the differences between proteins expressions among the different tissue types can be seen in the Supplementary data (Figure S1 and Table S2, respectively). Table 3 shows the association between CD147 and CD44 expressions and both MCT1 and MCT4, suggesting a role for these glycoproteins as chaperones for the lactate transporters.

MCT4, GLUT-1 and CAIX change their cellular localization from prostate primary tumour to metastasis

Figure 2 (2A, 2B and 2C) shows representative immunohistochemical reactions for all proteins in BT, PIN, TT and MT and Table 4 summarizes the sub-cellular localization of each protein studied in the different human tissues. In general, normal prostate epithelium was either negative or weakly positive for most of metabolic proteins studied with the exception for MCT1 CD147 and CD44, whereas PIN lesions and adenocarcinomas showed intense staining in most cases. The most strikingly findings were obtained for MCT4 (Fig 2A, C1-C4), GLUT-1 (Fig 2B,A1-A4) and CAIX (Fig. 2B, F1-F4) that change their subcellular localization to the plasma membrane TT to MT. Also HIF1-alpha appeared in the nucleus of the cells in metastatic samples (Fig. 2B, G4).

Proteins involved in glycolytic metabolism associate with poor prognosis parameters

The detailed associations between the expression of the metabolic proteins and clinico-pathological data is presented in Table 5. In general, we observed that various proteins involved in the glycolytic pathway associate with important prognostic parameters in contrast to proteins involved in fatty acid oxidation.

Importantly, MCT4 and CAIX, were the only ones showing an association with patients' survival status (p=0.040 and p=0.039, respectively). Kaplan-Meier curves and Log Rank test showed no associations between disease-free survival and proteins expression, however, although not statistically significant,

there was a decrease in the survival of patients with tumours expressing CAIX, (data not shown).

Different *in vitro* models of PCa progression exhibit different protein expression and glycolytic metabolism profiles

Since the main differences observed across malignant progression in human samples were related to proteins involved in the glycolytic metabolism, in Figure 3 we show the expression profile of these proteins in different *in vitro* models of disease progression. Interestingly, MCT4 and HIF-1alpha were only proteins detected in DU145 and PC3 cell lines. Also, GLUT-1, LDHV and CAIX showed evident higher levels in PC3 cell line. Interestingly, the ICC results for the different cell line models show that MCT4 and GLUT-1 only appeared evidently expressed at the plasma membrane in the highly metastatic models, such as PC3, in contrast to the low tumorigenic cell line LNCaP (Figure 3B).

Finally, a clear distinct metabolic behaviour between the low tumorigenic 22RV1 and LNCaP and the high tumorigenic PC3 and DU145 models was observed. PC3 and DU145 exhibited higher levels of glucose consumption (Figure 3C) and lactate production (Figure 3D) when compared to 22RV1 and LNCaP cells.

Prostate cancer cells exhibited different sensitivities to inhibition of glycolysis and fatty acid oxidation

Based in the idea that different models of disease progression exhibited a different metabolic behaviour, we aimed to study the effect of glycolysis and fatty acid oxidation inhibition in the different models.

Oxamic acid (OA), an inhibitor of lactic dehydrogenase (LDH), was used to infer how the glycolytic metabolism in the different models is affected after inhibition of lactic acid production. Figure 4 shows the glucose consumption and lactate production of prostate cell lines after the treatment with OA. It is possible to observe that both parameters showed a significant decrease in 22RV1, PC3 and DU145 cell lines. In contrast, LNCaP cell line metabolism was less affected by OA treatment.

The phenothiazine drug, thioridazine (TZ), described as a selective inhibitor of peroxisomal β-oxidation, was also used in our cell line models. As observed in Figure 5A, the IC_{50} values show that 22RV1 and LNCaP cell lines are more sensitive to TZ effect, especially at 72 h treatment. Accordingly, when we supplemented the culture media with phytanic acid (PA), a long branched chain fatty acid, to stimulate fatty acid oxidation, a decrease in PC3 and DU145 cell viability was observed, but not in 22RV1 and LNCaP that maintain their viability with increasing concentrations of PA, even after 48 hours of exposure (Figure 5B).

Ultrastructural differences are evident among the different prostate cancer cell models

So far, we have observed that differences at the expression levels of proteins involved in glycolytic and fatty acid metabolism as well as in cells metabolic behavior are evident across malignant transformation. As so, we also aimed to verify if such differences might be also reflected at the ultrastructural level of the cells. Figure 6 shows electron micrographs of LNCaP (Figure 6A), PC3 (Figure 6B) and two different human samples of prostate cancer (Figure 6C and 6D). It was possible to observe that either in LNCaP cells or the human samples (Figure 6A, 6C and 6D, respectively), cells are packed with lipid bodies. Also, in what concerns mitochondria morphology, these images clearly show differences in terms of subcellular features of localized prostate tumours and the low tumorigenic model LNCaP cell line in comparison with the cells derived from highly metastatic tumours (PC3).

Analysis of the mitochondrial genome reflects a decrease in the efficiency of PC3 cell lines through increased expression of UCP2 protein

A complete mtDNA sequencing of the cell lines LNCaP and PC3 (as two clearly distinct models of disease progression) was performed (Table 6). The results showed that the LNCaP haplotype can be affiliated in the European haplogroup H26c, displaying the coding private mutation in ND5 gene at position 13227, which is a synonymous polymorphism. The PC3 haplotype belongs to another European haplogroup, denominated U5a1c, and bears two non synonymous

recent mutations: in gene ND4, at position 11120, leading to the F121L amino acid replacement, which has a MutPred pathogenicity score of 0.423; and in gene ND5, at position *13802,* conducting to the amino acid replacement T489M, having a MutPred pathogenicity score of 0.6. We have inferred, on the basis of a large population dataset of mtDNA genomes, that the value of MutPred score dividing pathogenic mutations from tolerated mutations is around 0.7. The two nonsynonymous mutations in PC3 cell line have scores below this threshold, but we do not know yet if the mutations can contribute additively to a phenotype. As both PC3 mutations are located in proteins of the Complex I, if they have some functional impairment in the oxidative phosphorylation it will be upon this complex.

Investigating Complex I activity in prostate cell lines we observed that in contrast with we were expecting, PC3 cells show increased Complex I activity (Figure 7A). However the increased Complex I activity in PC3 cells was not reflected in global cellular ATP levels (Figure 7B). These results suggest that the proton electrochemical gradient across the inner mitochondrial membrane (IMM) is being dissipated and not used for ATP production. In fact, investigating the expression levels of the mitochondrial uncoupling protein 2 (UCP2), we verified that it is significantly higher in PC3 cells, both using β-actin protein ($p=0.02$) as total protein loading, and the translocase of outer mitochondrial membrane 20 homolog (TOMM20) ($p<0.01$), as a mitochondrial protein loading indicating that PC3 cells exhibit few mitochondria and thus confirming our ultrastructural data (Figure 8A-D).

Public genome-wide expression profiling datasets show association of metabolic-related proteins with disease progression

By making use of publicly available genome-wide expression profiling datasets, we immediately observed that the mRNA levels of genes involved in the glycolytic pathway were higher in metastatic tumours compared to localized tumours (Figure 9A), whereas, the differences in the expression patterns observed for the genes involved in the fatty acid oxidation pathway were less striking (Figure 9B).

Interestingly, looking to the genes that codify for lactate transporter proteins, only the mRNA levels of SLC16A3, the gene that codifies for MCT4, the isoform responsible for lactate extrusion were higher in metastatic compared to localized tumours (Figure 9E). Finally, the Glinsky dataset showed again SLC16A3 gene being associated with the time to biochemical recurrence after surgery (Figure 10).

Discussion

While numerous studies have investigated the involvement of altered cellular metabolism in various tumours, little is known about the metabolic alterations during prostate cancer progression.

Costello *et al*. [25,26] described the transformation of a citrate-producing epithelial cell to a malignant citrate oxidizing, which must be an early event for malignancy and progression. Additionally, Shan Zha *et al* described the selective upregulation of peroxisomal branched chain fatty acid β-oxidation pathway in prostate cancer [17], which might represent a good source of acetyl-CoA for Krebs cycle, and later, Ranasinghe W. *et al* [27] linked hypoxia with aggressiveness and metastasis in prostate cancer. However, whether the expression of proteins involved in cellular energetic metabolism is altered during prostate malignant transformation and progression, as well as, the significance of their expression is largely unknown.

In this study, we found a consistent overexpression of proteins involved in peroxisomal branched chain fatty acid oxidation in prostate cancer as well as in PIN lesions in contrast to benign glands, suggesting a possible etiological role of this pathway in malignant transformation. In the other hand, the expression of proteins involved in the glycolytic pathway showed association with reliable predictors of poor prognosis such as tumour stage (pT), Gleason score and biochemical recurrence, suggesting a role in disease progression.

Accordingly, our *in vitro* studies showed that different models of disease progression exhibited different metabolic profiles. The cell lines derived from distant metastasis are more glycolytic and strongly express MCT4 and GLUT-1

at the plasma membrane, two major players in the glucose uptake and lactate extrusion essential for the proper function of the glycolytic pathway. Importantly, a change in the subcellular localization of these proteins from localized tumour to metastatic tumour was verified, with MCT4, GLUT1 and CAIX being present at the plasma membrane only in metastatic samples, suggesting an obvious link between the plasma membrane function of these proteins and the aggressive stage of the tumour.

Using inhibitors of the different pathways we were able to support the idea that different metabolic pathways are active according to the disease stage of the tumour. Thus, targeting fatty acid oxidation seems to be more relevant in an early stage in contrast to the glycolytic pathway, which will produce more effects in an advanced stage of the disease.

Additionally, differences were also evident at the organelle content level, between low and high tumorigenic models, which led us to investigate the idea of a less energetically efficient respiration of the highly tumorigenic models. In fact, we found an false increase in Complex I activity in PC3 cells masked by the overexpression of UCP2 protein, justifying a coupling defect in oxidative phosphorylation in these cells. Interestingly, a coupling defect in oxidative phosphorylation was also described in mitochondrion-rich (Hürthle or oncocytic) tumours of the thyroid [28-31].

Finally, publicity dataset supported our main findings showing that the expression of genes involved in fatty acid oxidation mostly changed from benign samples to localized tumour specimens, suggesting a clear role mainly in prostate malignant transformation rather than progression, whereas, genes involved in the glycolytic metabolism are aberrantly expressed mainly in the metastatic samples, being more compatible with a role in prostate cancer progression and aggressiveness, and drawing attention in this case for SLC16A3 gene that codifies MCT4 as a predictive marker of disease progression and aggressiveness. This data also indicates that prostate cancer does not fit in the traditional model of a metabolic switch to glycolysis from non malignant to malignant cells but instead this metabolic switch is more likely to

be involved in the advanced stage of the disease and importantly prior to pathologic transformation, exhibiting a predictive value.

To the best of our knowledge, this is the largest study investigating the role of key metabolic-related proteins in relation to outcome of radical treatment to localized prostate cancer, putting together a large variety of clinical samples and *in vitro* models, demonstrating the metabolic heterogeneity of prostate cancer and its significance as diagnostic, prognostic and therapeutic tool in prostate cancer disease.

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Statement of author contributions

All authors meet the criteria for authorship in that they have participated in the conception, execution or interpretation of at least part of the publication in their field of expertise.

All authors read and approved the final manuscript.

References

1. Warburg, O., F. Wind, and E. Negelein, *The Metabolism of Tumors in the Body.* J Gen Physiol, 1927. **8**(6): p. 519-30.
2. Miranda-Goncalves, V., et al., *Monocarboxylate transporters (MCTs) in gliomas: expression and exploitation as therapeutic targets.* Neuro Oncol, 2013. **15**(2): p. 172-88.
3. Pinheiro, C., et al., *Role of monocarboxylate transporters in human cancers: state of the art.* J Bioenerg Biomembr, 2012. **44**(1): p. 127-39.
4. Pinheiro, C., et al., *Expression of monocarboxylate transporters 1, 2, and 4 in human tumours and their association with CD147 and CD44.* J Biomed Biotechnol, 2010. **2010**: p. 427694.
5. Pinheiro, C., et al., *Monocarboxylate transporters 1 and 4 are associated with CD147 in cervical carcinoma.* Dis Markers, 2009. **26**(3): p. 97-103.
6. Pinheiro, C., et al., *Increasing expression of monocarboxylate transporters 1 and 4 along progression to invasive cervical carcinoma.* Int J Gynecol Pathol, 2008. **27**(4): p. 568-74.
7. Pinheiro, C., et al., *Increased expression of monocarboxylate transporters 1, 2, and 4 in colorectal carcinomas.* Virchows Arch, 2008. **452**(2): p. 139-46.
8. Pertega-Gomes, N., et al., *Monocarboxylate transporter 2 (MCT2) as putative biomarker in prostate cancer.* Prostate, 2012.
9. Pertega-Gomes, N., et al., *Monocarboxylate transporter 4 (MCT4) and CD147 overexpression is associated with poor prognosis in prostate cancer.* BMC Cancer, 2011. **11**: p. 312.
10. Higgins, L.H., et al., *Hypoxia and the metabolic phenotype of prostate cancer cells.* Biochim Biophys Acta, 2009. **1787**(12): p. 1433-43.
11. Effert, P., et al., *Expression of glucose transporter 1 (Glut-1) in cell lines and clinical specimens from human prostate adenocarcinoma.* Anticancer Res, 2004. **24**(5A): p. 3057-63.
12. Stewart, G.D., et al., *Analysis of hypoxia-associated gene expression in prostate cancer: lysyl oxidase and glucose transporter-1 expression correlate with Gleason score.* Oncol Rep, 2008. **20**(6): p. 1561-7.
13. Vaz, C.V., et al., *Androgen-responsive and nonresponsive prostate cancer cells present a distinct glycolytic metabolism profile.* Int J Biochem Cell Biol, 2012. **44**(11): p. 2077-84.
14. Giovacchini, G., et al., *[11C]choline positron emission tomography/computerized tomography to restage prostate cancer cases with biochemical failure after radical prostatectomy and no disease evidence on conventional imaging.* J Urol, 2010. **184**(3): p. 938-43.
15. Hong, H., et al., *Positron emission tomography imaging of prostate cancer.* Amino Acids, 2010. **39**(1): p. 11-27.

16. Farsad, M., et al., *Positron-emission tomography in imaging and staging prostate cancer.* Cancer Biomark, 2008. **4**(4-5): p. 277-84.
17. Zha, S., et al., *Peroxisomal branched chain fatty acid beta-oxidation pathway is upregulated in prostate cancer.* Prostate, 2005. **63**(4): p. 316-23.
18. Liu, Y., L.S. Zuckier, and N.V. Ghesani, *Dominant uptake of fatty acid over glucose by prostate cells: a potential new diagnostic and therapeutic approach.* Anticancer Res, 2010. **30**(2): p. 369-74.
19. Lobo-da-Cunha, A., et al., *Histochemical and ultrastructural characterization of the posterior esophagus of Bulla striata (Mollusca, Opisthobranchia).* Microsc Microanal, 2010. **16**(6): p. 688-98.
20. Andrews, R.M., et al., *Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA.* Nat Genet, 1999. **23**(2): p. 147.
21. Pereira, L., et al., *The diversity present in 5140 human mitochondrial genomes.* Am J Hum Genet, 2009. **84**(5): p. 628-40.
22. Li, B., et al., *Automated inference of molecular mechanisms of disease from amino acid substitutions.* Bioinformatics, 2009. **25**(21): p. 2744-50.
23. Pereira, L., et al., *Comparing phylogeny and the predicted pathogenicity of protein variations reveals equal purifying selection across the global human mtDNA diversity.* Am J Hum Genet, 2011. **88**(4): p. 433-9.
24. Kloss-Brandstatter, A., et al., *HaploGrep: a fast and reliable algorithm for automatic classification of mitochondrial DNA haplogroups.* Hum Mutat, 2011. **32**(1): p. 25-32.
25. Costello, L.C. and R.B. Franklin, *The clinical relevance of the metabolism of prostate cancer; zinc and tumor suppression: connecting the dots.* Mol Cancer, 2006. **5**: p. 17.
26. Costello, L.C., et al., *Zinc and prostate cancer: a critical scientific, medical, and public interest issue (United States).* Cancer Causes Control, 2005. **16**(8): p. 901-15.
27. Ranasinghe, W.K., et al., *The role of hypoxia-inducible factor 1alpha in determining the properties of castrate-resistant prostate cancers.* PLoS One, 2013. **8**(1): p. e54251.
28. Maximo, V., et al., *The biology and the genetics of Hurthle cell tumors of the thyroid.* Endocr Relat Cancer, 2012. **19**(4): p. R131-47.
29. Maximo, V., et al., *Mitochondrial DNA somatic mutations (point mutations and large deletions) and mitochondrial DNA variants in human thyroid pathology: a study with emphasis on Hurthle cell tumors.* Am J Pathol, 2002. **160**(5): p. 1857-65.
30. Savagner, F., et al., *Defective mitochondrial ATP synthesis in oxyphilic thyroid tumors.* J Clin Endocrinol Metab, 2001. **86**(10): p. 4920-5.
31. Gasparre, G., et al., *Disruptive mitochondrial DNA mutations in complex I subunits are markers of oncocytic phenotype in thyroid tumors.* Proc Natl Acad Sci U S A, 2007. **104**(21): p. 9001-6.

Tables

Table 1. Details of the immunohistochemical procedure used to analyze the expression of the different proteins.

Protein	Antibody	Company	Antibody Dilution	Positive Control	Incubation Period	Detection System
MCT4	sc-50329	Santa Cruz Biotechnology	1:500	Colon tumor	Overnight	R.T.U. Vectastain Universal Elite ABC Kit, Vector, EUA
MCT2	sc-50322	Santa Cruz Biotechnology	1:200	Muscle	2 hours	Ultravision Detection System Anti-polyvalent, HRP, Labvision Corporation, Freemont, CA
CD44	156-3C11	Serotec	1:1000	Colon tumor		
GLUT1	ab 15309	Abcam	1:2000	Head and neck tumor		
CAIX	ab 15086	Abcam	1:2000	Stomach		
MCT1	sc-365501	Santa Cruz Biotechnology	1:500	Colon tumor	Overnight	R.T.U. Vectastain Universal Elite ABC Kit, Vector, EUA
HIF-1α	610958	BD Biosciences	1:100	Glioblastoma		
GLUT12	ab 75441	Abcam	1:500	Rim	Overnight	Ultravision Detection System Anti-polyvalent, HRP, Labvision Corporation, Freemont, CA
LDHV	ab 53010	Abcam	1:1000	Colon tumor	2 hours	
HKII	ab104836	Abcam	1:750	Colon tumor		
PDK1	ab110025	Abcam	1:500	Stomach		
CD147	sc-71038	Santa Cruz Biotechnology	1:400	Colon tumor		
AMACR	504R-16	Cell Marque	1:50	Kidney		
ACOX3	sc-135435	Santa Cruz Biotechnology	1:250	Liver		
DBP	DBP antibody was a gift from Dr. Gabriele Moller from HelmholtzZentrum mÜnchen.		Ready to use	Kidney		

Table 2. Details of the Western-blot procedure used to analyze the expression of the different proteins.

	Primary Antibody						
Protein Marker	**Reference**	**Company**	**Antibody Dilution**	**Incubation Period**	**Reactivity**	**Reference**	**Company**
CD44	156-3C11	Serotec	1:1000	overnight	anti-mouse	sc-2031	Santa Cruz Biotechnology
MCT1	sc-365501	Santa Cruz Biotechnology	1:500				
HIF-1α	610958	BD Biosciences	1:1000				
HKII	ab104836	Abcam	1:2000				
PDK1	ab110025	Abcam	1:2000				
CD147	sc-71038	Santa Cruz Biotechnology	1:100				
MCT4	sc-50329	Santa Cruz Biotechnology	1:500		anti-rabbit	sc-2004	
GLUT1	ab 15309	Abcam	1:800				
CAIX	ab 15086	Abcam	1:2000				
LDHV	ab 53010	Abcam	1:2000				
TOM20	sc-11415	Santa Cruz Biotechnology	1:5000		anti-rabbit		
UCP-2	sc-6525	Santa Cruz Biotechnology	1:400		anti-goat		
Actin	sc-1616	Santa Cruz Biotechnology	1:5000		anti-goat	sc-2020	

Table 3. Association between MCT1, MCT2, MCT4, CD147 and CD44 expressions in prostate tumours.

		MCT1		***MCT4***		***MCT2***	
	n	**Positive (%)**	***p***	**Positive (%)**	***p***	**Positive (%)**	***p***
CD147			**<0.001**		**<0.001**		0.411
Negative	**283**	39.6		6.2		34.7	
Positive	**185**	60.4		17.0		36.5	
CD44			**<0.001**		**0.003**		**0.002**
Negative	**274**	35.8		7.3		21.8	
Positive	**126**	53.2		17.1		37.2	

Table 4. Details of proteins expression regarding subcellular localization.

	Proteins sub- cellular Localization			
	BT	**PIN**	**TT**	**MT**
Proteins				
MCT1	PM (Fig. 2A,A1)	PM (Fig. 2A,A2)	PM (Fig. 2A,A3)	PM (Fig. 2A,A4)
MCT2	C (Fig. 2A,B1)	C (Fig. 2A,B2)	C (Fig. 2A,B3)	C (Fig. 2A,B4)
MCT4	**C (Fig. 2A,C1)**	**C (Fig. 2A,C2)**	**C (Fig. 2A,C3)**	**PM (Fig. 2A,C4)**
CD147	PM (Fig. 2A,D1)	PM (Fig. 2A,D2)	PM (Fig. 2A,D3)	PM(Fig. 2A,D4)
CD44	PM (Fig. 2A,E1)	PM (Fig. 2A,E2)	PM (Fig. 2A,E3)	PM(Fig. 2A,E4)
GLUT1	**C (Fig. 2B,A1)**	**C (Fig. 2B,A2)**	**C (Fig. 2B,A3)**	**PM (Fig. 2B,A4)**
GLUT12	C (Fig. 2B,B1)	C (Fig. 2B,B2)	C (Fig. 2B,B3)	C (Fig. 2B,B4)
LDHV	C (Fig. 2B,C1)	C (Fig. 2B,C2)	C (Fig. 2B,C3)	C (Fig. 2B,C4)
HKII	C (Fig. 2B,D1)	C (2B,B2)	C (2B,B2)	C (2B,B2)
PDK1	C (Fig. 2B,E1)	C (2B,E2)	C (2B,E3)	C (2B,E4)
CAIX	**C (Fig. 2B,F1)**	**C (Fig. 2B,F2)**	**C (Fig. 2B,F3)**	**PM (Fig. 2B,F4)**
HIF	**NS**	**NS**	**N (Fig. 2B,G3)**	**N (Fig. 2B,G4)**
AMACR	C (Fig. 2C,A1)	C (Fig. 2C,A2)	C (Fig. 2C,A3)	C (Fig. 2C,A4)
ACOX3	C (Fig. 2C,B1)	C (Fig. 2C,B2)	C (Fig. 2C,B3)	C (Fig. 2C,B4)
DBP	C (Fig. 2C,C1)	C (Fig. 2C,C2)	C (Fig. 2C,C3)	C (Fig. 2C,C4)

(NS) No staining. Staining at plasma membrane (PM), cytoplasm (C) or nucleus (N) in different prostate tissues.

Table 5. Correlations between key metabolic-related proteins expressions in prostate tumour samples and clinico-pathological data.

		MCT1			MCT2			MCT4			CD147			
Variable	**n**	**ISG %**	***p***	**HSG %**	***p***	**ISG %**	***p***	**HSG %**	***p***	**ISG %**	***p***	**HSG %**	***p***	**ISG %**	***p***	**HSG %**	***p***
Age			0.309		0.114		0.322		0.106		0.212		0.144		**0.008**		0.282
□ 64	**250**	17.6		27.2		32.2		45.8		5.9		3.1		14.1		19.7	
>64	**220**	15.5		32.7		26.4		59.3		3.7		5.8		23.3		22.3	
PSA (ng/ml)			**0.006**		0.268		0.339		0.256		0.219		0.057		0.298		0.450
□ 9.4	**243**	14.0		32.5		26.8		56.1		5.2		3.3		17.8		21.9	
>9.4	**122**	25.4		28.7		32.1		48.2		8.1		8.1		20.7		20.7	
leason score			**0.014**		0.074		0.863		0.516		0.362		0.508		**0.001**		**0.002**
<7	**142**	10.6		37.3		28.1		43.8		2.7		2.7		29.1		10.6	
7	**290**	20.0		27.6		30.7		55.3		6.0		5.3		14.5		24.8	
>7	**31**	6.5		22.6		20.0		60.0		3.3		3.3		9.7		29.0	
Primary			0.462		**0.001**		0.303		0.510		0.555		0.462				
eason Pattern															**0.000**		**0.035**
2 or 3	**384**	16.4		33.1		28.0		52.7		4.8		4.2		21.5		19.1	
4 or 5	**86**	17.4		16.3		36.8		50.0		5.1		5.1		5.8		28.7	
Secondary			0.081		0.499		0.439		0.121		0.181		0.291				
eason Pattern															**0.021**		**0.005**
2 or 3	**210**	13.3		30.5		31.3		44.9		3.5		3.5		23.0		15.2	
4 or 5	**253**	18.6		30.0		28.1		57.8		6.0		5.1		15.1		25.4	
pT			0.344		0.059		0.115		0.268		0.288		**0.030**		0.157		**0.030**
2	**357**	16.5		31.9		32.2		50.0		4.5		3.2		19.4		19.0	
3	**99**	14.1		23.2		18.5		59.3		6.6		8.8		14.3		28.6	
Perineural			0.064		0.254		0.292		0.300		0.225		0.286				
Invasion															0.117		0.169
Absent	**126**	21.4		27.0		24.2		57.6		6.7		2.9		22.4		17.6	
Present	**342**	14.9		30.7		31.6		50.0		4.2		4.9		17.0		22.2	
Biochemical			0.427		0.161		0.485		0.389		0.626		**0.003**				
Recurrence															0.078		0.224
Absent	**404**	16.8		29.0		30.3		50.6		4.9		2.9		19.7		20.1	
Present	**67**	14.9		35.8		27.8		55.6		4.7		12.3		11.8		25.0	
urvival Status			0.279		0.351		0.427		0.125		**0.040**		0.731		0.235		0.162
No	**7**	0		42.9		16.7		83.3		28.6		0.0		0		42.9	
Yes	**463**	16.8		29.7		30.2		50.5		4.4		4.4		18.8		20.5	

		CD44		GLUT1		GLUT12		LDHV		HKII		PDK1	
Variable	**n**	HSG %(n)	***p***	HSG %(n)	***p***	HSG %(n)	***p***	HSG %(n)	***p***	HSG %(n)	***p***	HSG %(n)	***p***
Age			**0.017**		0.434		0.102		**0.009**		0.358		0.380
≤ 64	**250**	26.6		21.9		41.8		23.2		14.9		42.0	
>64	**220**	37.0		22.9		48.6		33.5		16.5		40.2	
PSA (ng/ml)			0.343		0.387		0.303		0.449		0.103		**0.004**
≤9.4	**243**	32.2		22.8		47.2		31.3		17.4		45.5	
>9.4	**122**	29.2		20.8		43.4		29.9		11.7		30.6	
Gleason score			0.596		**0.000**		**0.000**		0.064		**0.001**		**0.000**
<7	**142**	27.8		11.4		26.4		20.6		6.3		23.2	
7	**290**	33.1		28.2		49.8		31.1		20.2		49.8	
>7	**31**	29.6		16.1		63.0		23.3		16.1		45.2	
Primary									0.168				
Gleason Pattern			**0.041**		0.382		0.079				0.157		0.346
2 or 3	**384**	33.5		22.0		42.9		29.1		14.5		40.7	
4 or 5	**86**	22.8		24.1		52.6		23.3		19.5		43.7	
Secondary									**0.007**				
Gleason Pattern			**0.026**		**0.002**		**0.000**				**0.007**		**0.000**
2 or 3	**210**	25.9		15.9		34.3		21.5		11.0		30.0	
4 or 5	**253**	35.6		27.6		51.8		32.2		19.6		50.8	
pT			**0.031**		0.066		0.217		0.405		0.357		
2	**357**	33.9		20.7		42.9		27.7		16.4		40.4	0.405
3	**99**	22.6		28.6		48.3		25.8		14.1		42.4	
Perineural													
Invasion			0.325		0.346				0.345		**0.037**		
Absent	**126**	33.7	.	20.3		32.7	**0.003**	26.0		10.4		33.9	**0.031**
Present	**342**	30.6		22.6		49.0		28.5	.	17.6		44.0	
Biochemical													
Recurrence			**0.050**		0.442		0.142		0.094		0.497		0.500
Absent	**404**	33.0		23.4		46.1		26.8		15.8		46.5	
Present	**67**	21.9		25.0		37.7		40.7		14.7		44.4	
Survival Status			0.619		0.478		0.613		0.540		0.7		0.606
No	**7**	28.6		14.3		42.9		33.3		14.3		42.9	
Yes	**463**	31.3		23.8		44.8		28.1		15.6		41.1	

		CAIX		AMACR		ACOX3				DBP			
Variable	**n**	ISG %(n)	***p***	HSG %(n)	***p***	ISG %(n)	***p***	HSG %(n)	***p***	ISG %(n)	***p***	HSG %(n)	***p***
Age			0.213		0.1		0.386		0.270		0.428		0.233
≤ 64	**250**	4.6		80.5		2.8		42.6		1.2		28.0	
>64	**220**	6.8		74.2		3.7		45.9		1.8		31.5	
PSA (ng/ml)			0.210		0.261		0.620		**0.035**		0.429		0.132
≤ 9.4	**243**	4.3		80.6		4.1		46.9		1.7		30.2	
>9.4	**122**	7.0		76.6		4.1		36.4		2.5		24.0	
Gleason score			0.536		0.081		0.098		0.195		0.063		0.343
<7	**142**	3.7		69.4		0.7		46.8		1.4		33.8	
7	**290**	6.3		79.0		4.2		44.1		1.0		28.4	
>7	**31**	6.7		88.9		6.5		29.0		6.5		22.6	
Primary					0.281		0.299		**0.015**		0.615		0.193
Gleason Pattern			0.166										
2 or 3	**384**	5.0		77.0		2.9		46.7		1.6		30.7	
4 or 5	**86**	8.4		81.1		4.6		33.3		1.1		25.3	
Secondary					0.167		0.108		0.239		0.599		0.194
Gleason Pattern			0.426										
2 or 3	**210**	5.9		74.6		1.9		41.9		1.4		31.9	
4 or 5	**253**	5.1		79.7		4.4		45.6		1.6		27.8	
pT			0.359		0.538		0.420		0.372		0.472		0.386
2	**357**	6.0		77.6		3.1		43.0		1.4		29.2	
3	**99**	4.2		77.9		4.0		45.5		2.0		31.3	
Perineural Invasion			0.555		**0.014**		0.395		0.527		0.598		0.351
Absent	**126**	5.7		67.5		2.4		43.7		1.6		31.2	
Present	**342**	5.6		80.4		3.5		43.9		1.5		28.9	
Biochemical Recurrence			0.468		0.353		0.375		**0.036**		0.732		0.550
Absent	**404**	5.5		76.9		3.0		45.8		1.5		29.6	
Present	**67**	6.5		80.4		4.4		33.3		1.5		29.4	
Survival Status			**0.039**		**0.049**		0.795		0.336		0.9		0.657
No	**7**	33.3		42.9		0		28.6		0		28.6	
Yes	**463**	5.2		78.1		3.3		44.2		1.5		29.6	

Table 6. mtDNA haplogroup affiliation of LNCaP and PC3 cell lines. All substitutions are transitions, and nomenclature reflects comparison versus the revised Cambridge Reference Sequence.

Cell line	Haplogroup	Polymorphisms characteristic of the haplogroup	Private polymorphisms	Reverted polymorphisms
LNCaP	H26c	146-263-750-1438-4769-8860-10562-11152	13227-16129-16519	15326
PC3	U5a1c	73-263-750-1438-2706-3197-4769-7028-8860-9477-11467-11719-12308-12372-13617-14766-14793-15218-15326-16192-16256-16270-16320-16399	195*-11120-*13802**-16172	

* These polymorphisms are characteristic of a very rare sub-haplogroup U5a1c1.

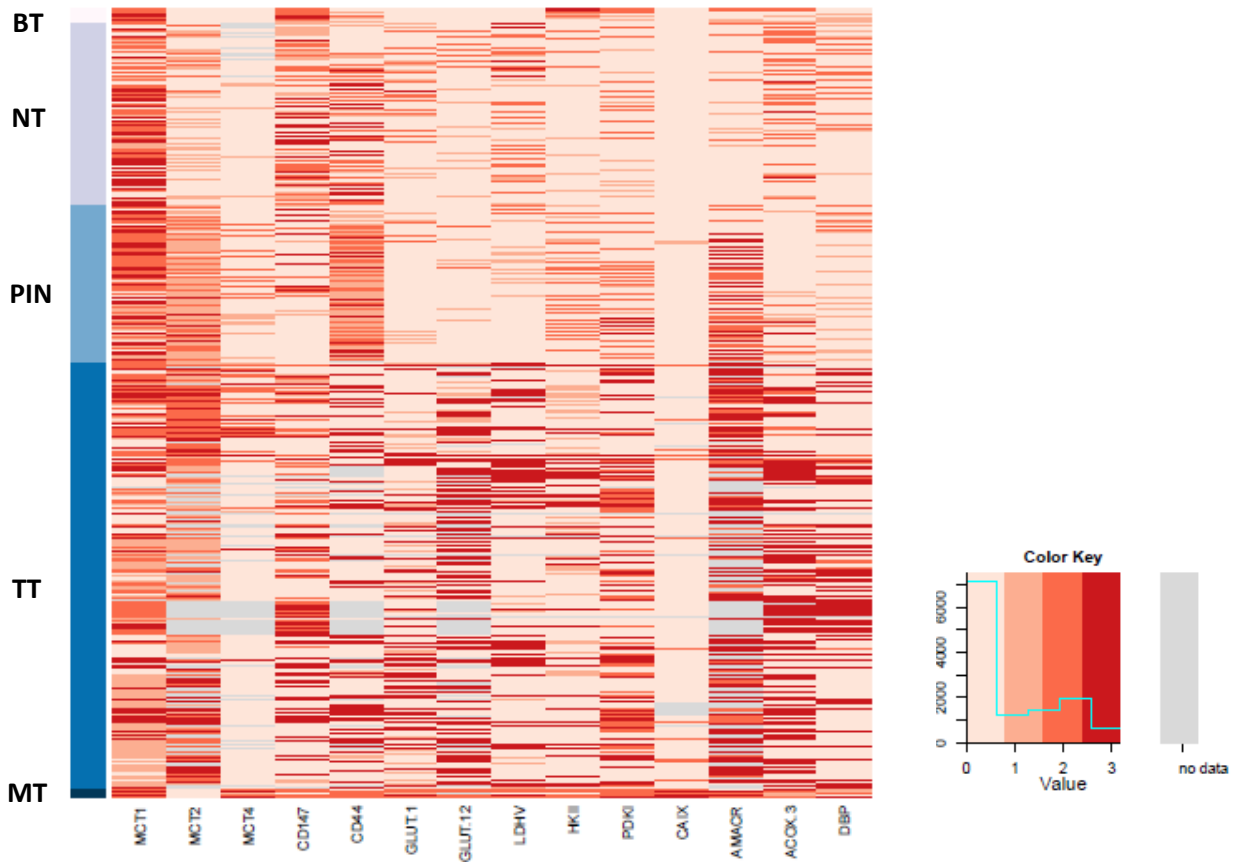
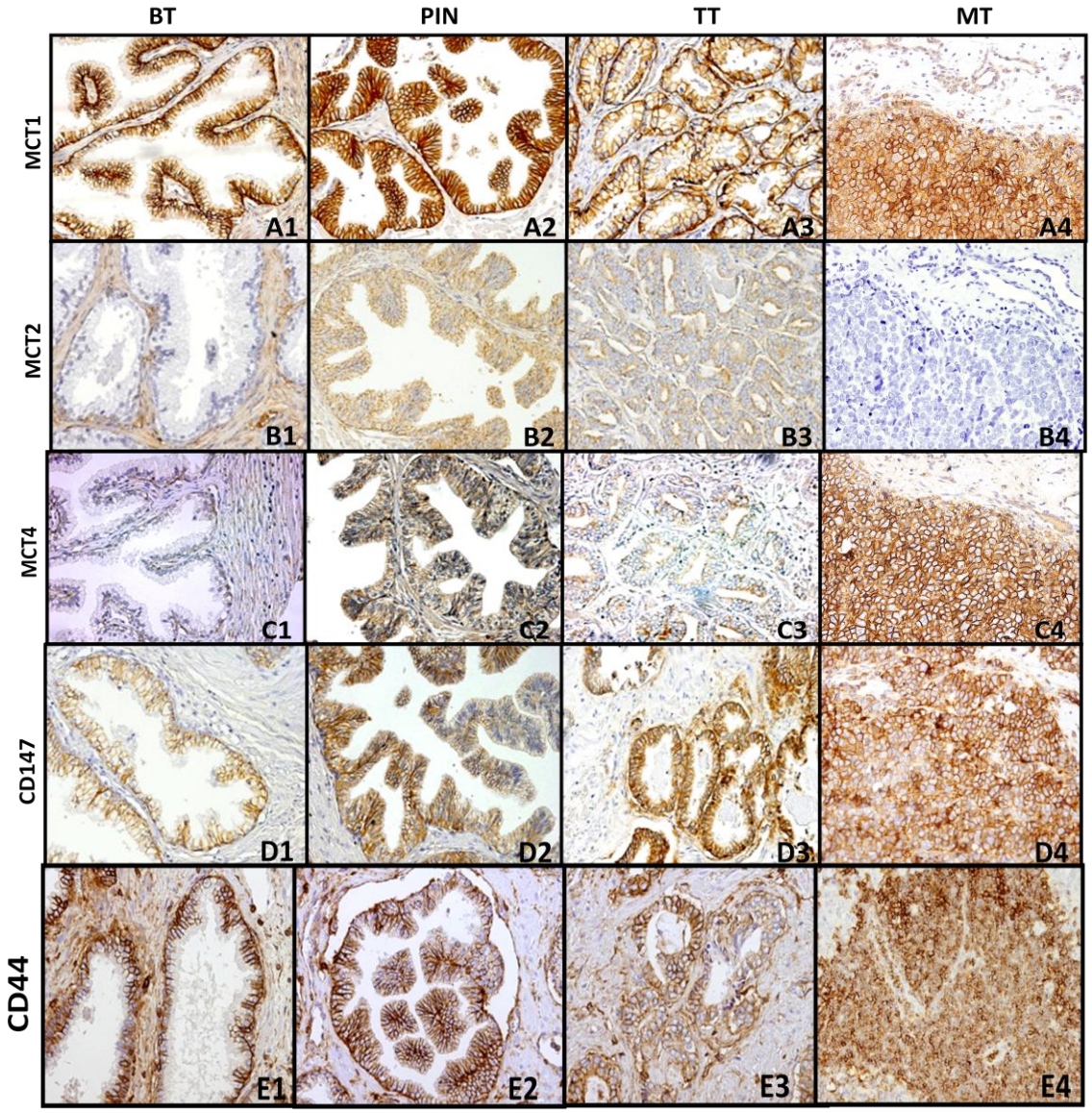
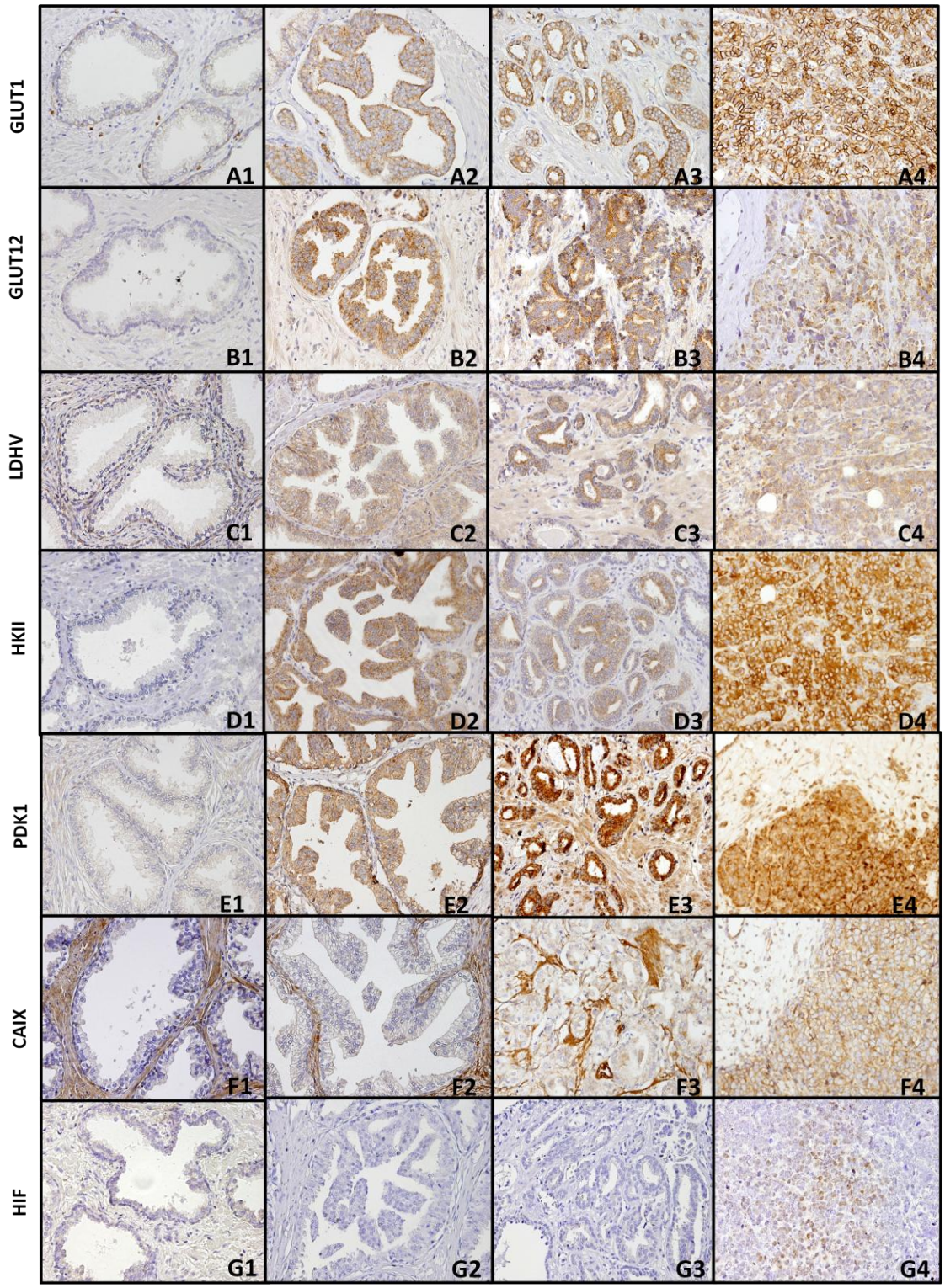


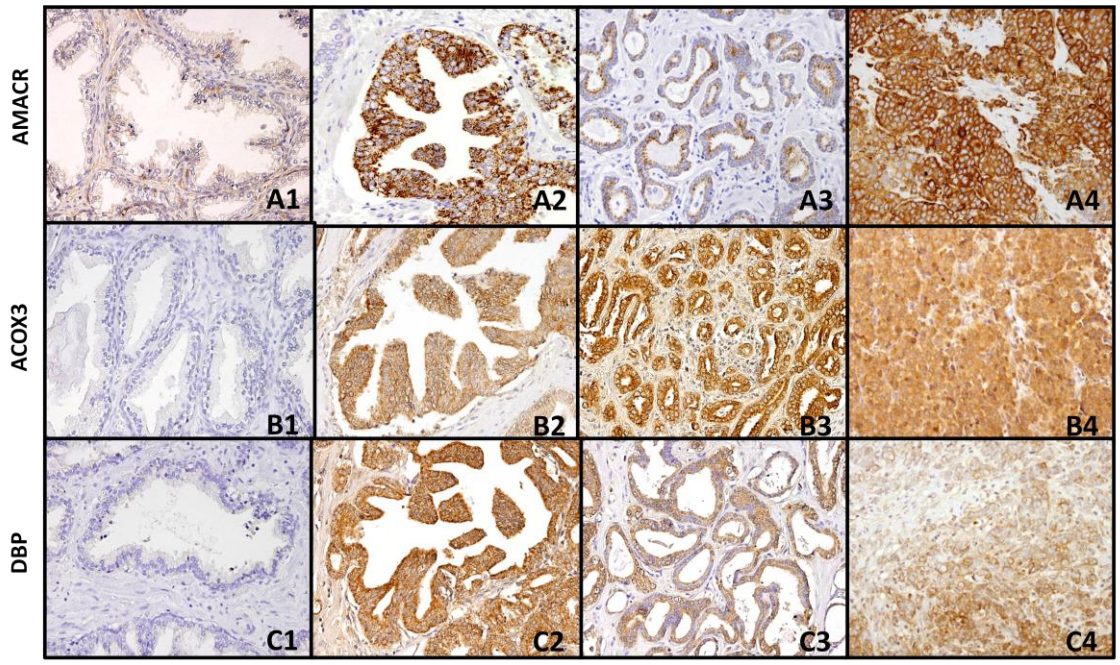
Figure 1. Heat map showing the groups benign tissue (BT), non-neoplastic tissue (NT), PIN lesions (PIN), tumour tissue (TT) and metastatic tissue (MT). The groups are red-colourcoded to the left of the heatmap, and protein names are given below each column.


BT
PIN
TT
MT
MCT1
MCT2
MCT4
CD147
CD44
A1
A2
A3
A4
B1
B2
B3
B4
C1
C2
C3
C4
D1
D2
D3
D4
E1
E2
E3
E4

(A)

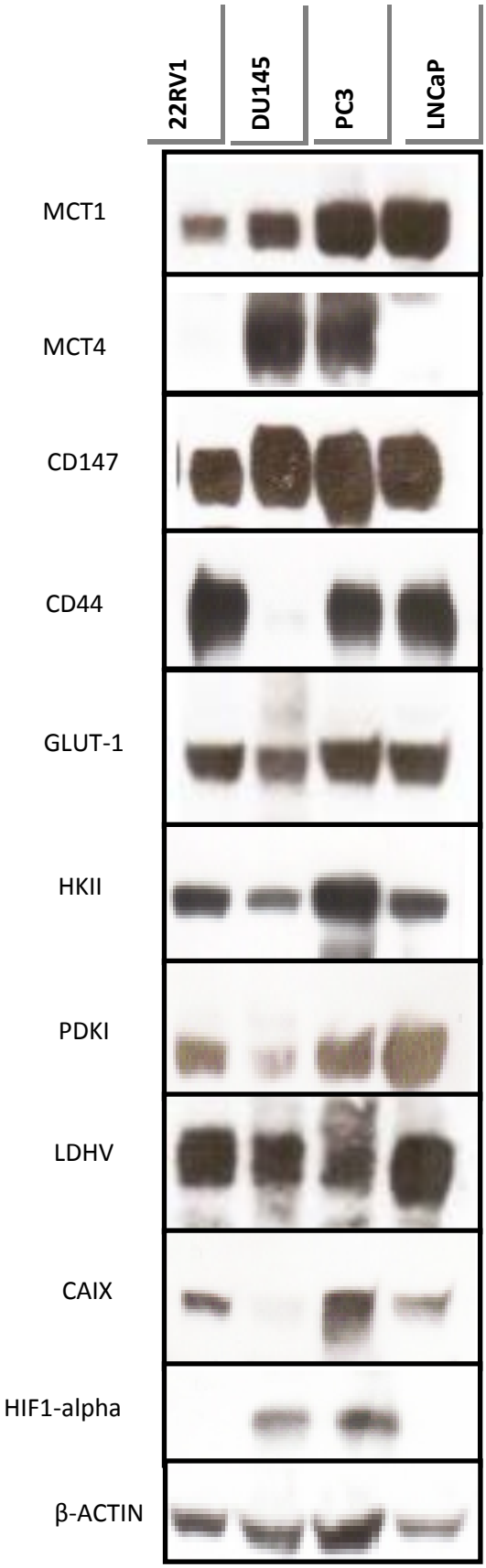

GLUT1
GLUT12
LDHV
HKII
PDK1
CAIX
HIF
A1
A2
A3
A4
B1
B2
B3
B4
C1
C2
C3
C4
D1
D2
D3
D4
E1
E2
E3
E4
F1
F2
F3
F4
G1
G2
G3
G4

(B)

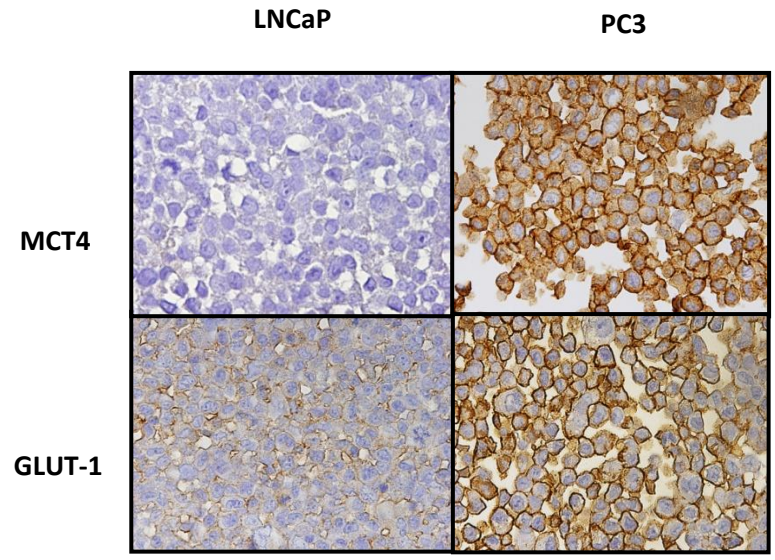


(C)

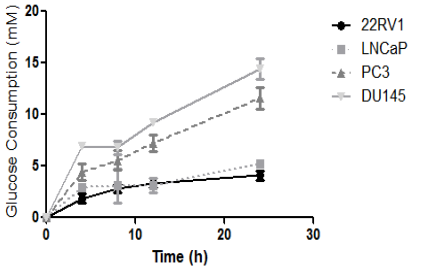
Figure 2. Immunohistochemical expression of metabolic-related proteins in benign tissue (BT) PIN lesions (PIN), prostate tumour tissue (TT) and metastatic tissue (MT) (200x magnification).



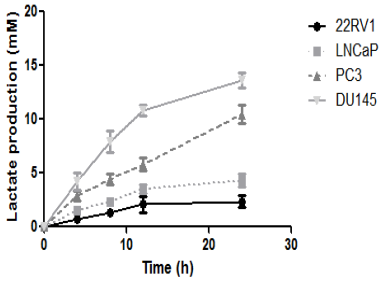
A)



B)



C)



D)

Figure 3. (A) Western blot analysis of metabolic-related proteins showing different levels of expression in different prostate cancer cell lines. (B) Immunohistochemical expression of MCT4 and GLUT-1 in LNCaP and PC3 cell lines and metabolic characterization of prostate cell lines based on glucose consumption (C) and lactate production (D).

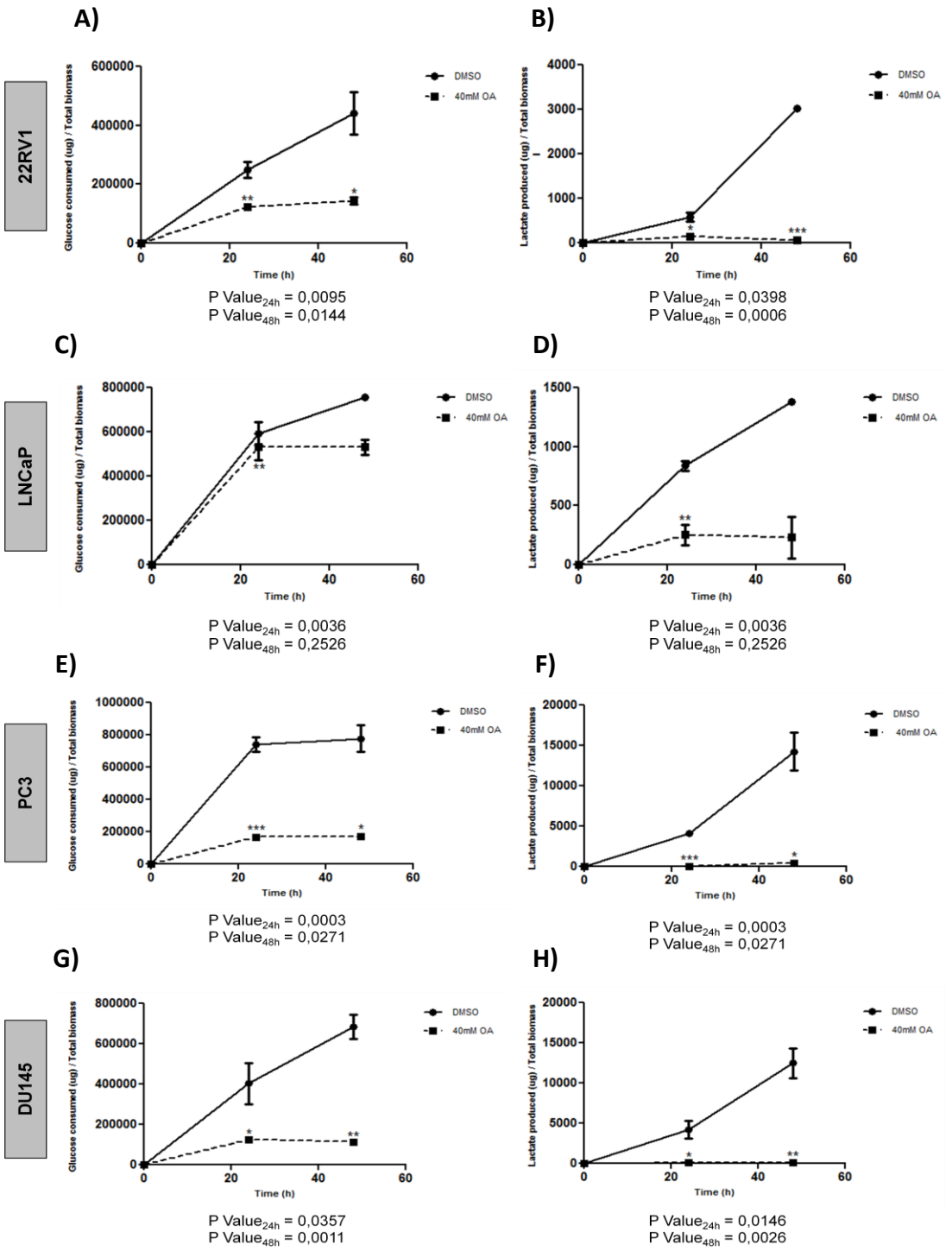


Figure 4. Effect of oxamic acid (OA) on cellular metabolism. The effect of OA on cellular metabolism was evaluated by extracellular glucose and lactate measurements. Glucose consumption and lactate production in 22RV1, LNCaP, PC3 and DU145 cell lines after the treatment with 40 mM of oxamic acid. Results were normalized to total biomass, at each time point. *p* values of the differences in the glucose consumption and lactate production were calculated in comparision with the control.

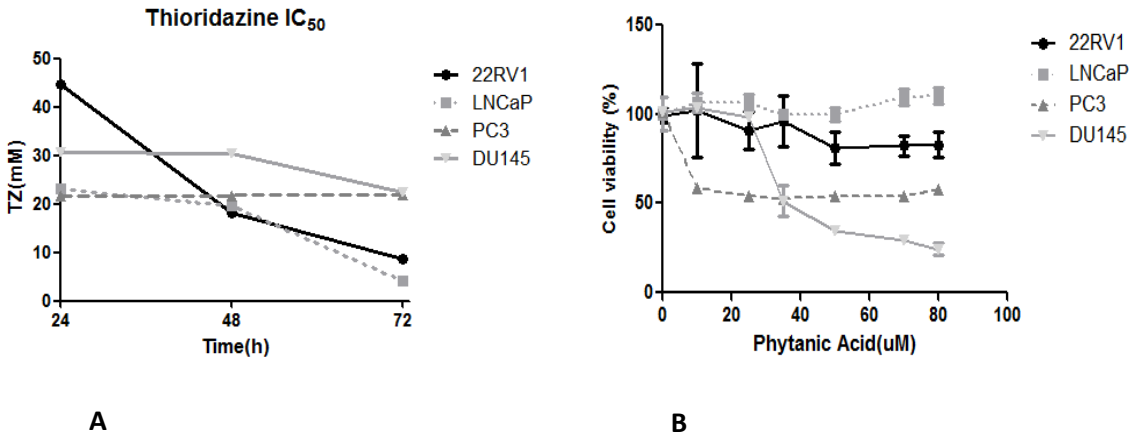
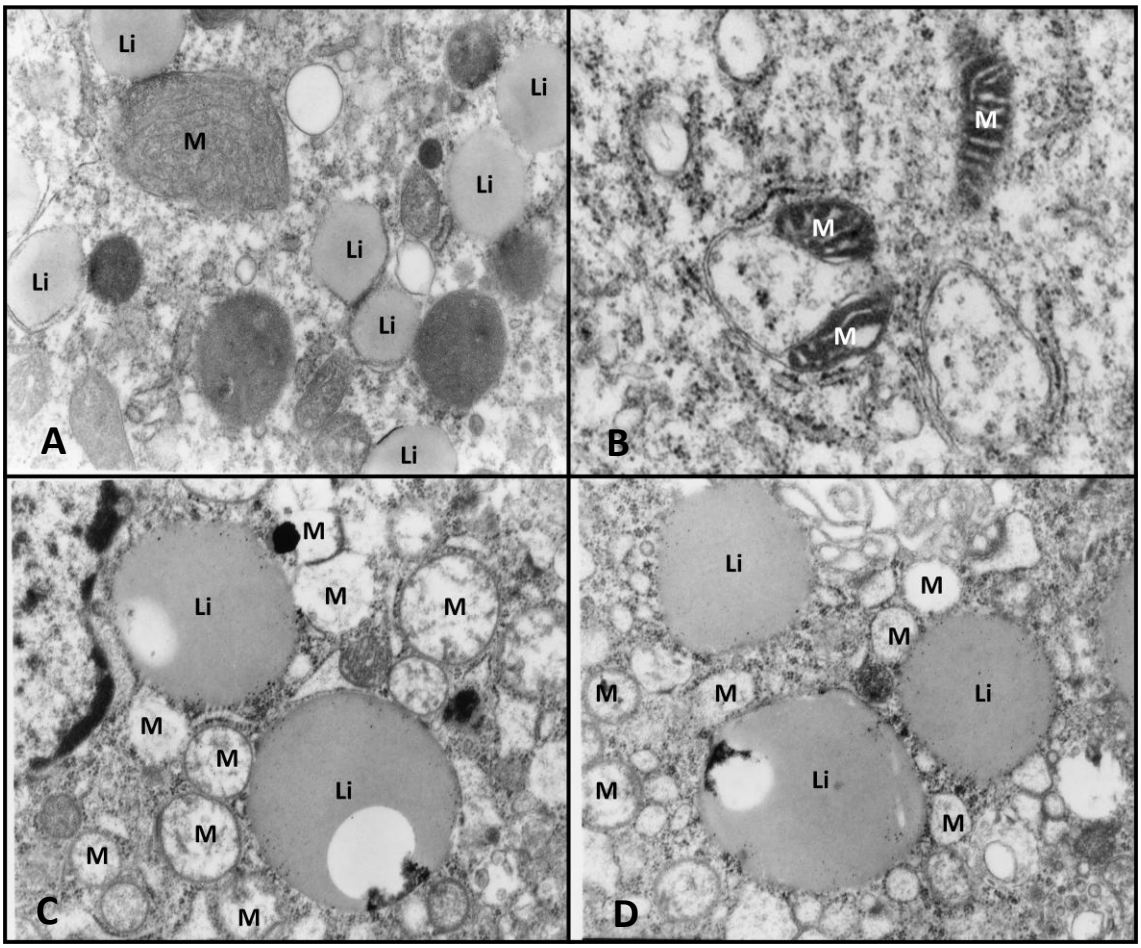


Figure 5. (A) Effect of thioridazine (TZ) in different prostate cancer cell lines. IC50 calculated for TZ in the different cell lines at different time points (24, 48 and 72 hours) is presented. (B) 22RV1, LNCaP, PC3 and DU145 cells viability (%) after 48 hours of exposure to increasing concentrations of phytanic acid.



M: Mitochondria; Li: Lipidic inclusions

Figure 6. Prostate cancer cells at a structural level. LNCaP (Figure 6A) and PC3 (Figure 6B) cell lines are represented, respectively. In LNCaP cells many mitochondria (M) can be observed and tend to be localized close to lipidic inclusions. Several lipidic (Li) droplets are seen randomly distributed into the cytoplasm of cells. There is a moderate amount of granular endoplasmic reticulum and free ribosomes (Ri). Similar to LNCaP are the cells collected from prostate localized tumour (Figure 6C and 6D). In contrast, in PC3 cells (6B), small atypical mitochondria (M) were observed as well as many free ribosomes (Ri), but with no lipidic inclusions.

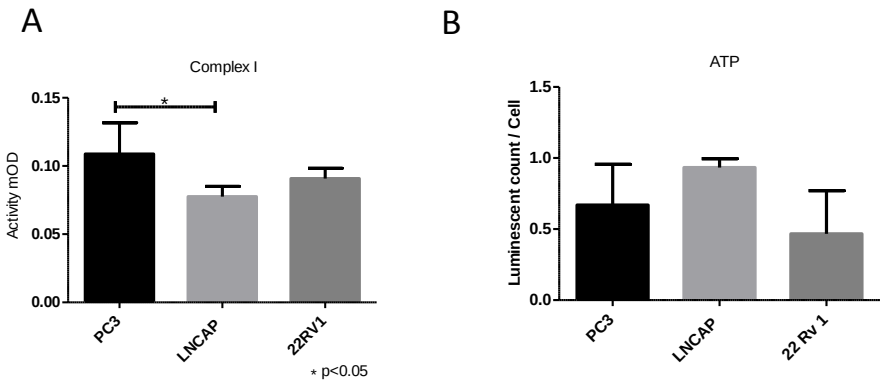


Figure 7. Analysis of Complex I activity. The activity of Complex I is proportional to the increase in absorbance at 450 nm. Activity mOD (milli-units of optical density) indicates that activity was measure at 450nm; Data correspond to two biological replicas done in duplicates. Bars, standard deviation; columns, mean. The asterisks indicates the level of statistical significance (*p<0.05); Data were subjected to one-way ANOVA and a posterior Tukey test. B) Analysis of global ATP levels. Data correspond to three biological experiments done in triplicates. Bars, standard deviation; columns, mean. Data were subjected to one-way ANOVA and posterior Tukey test.

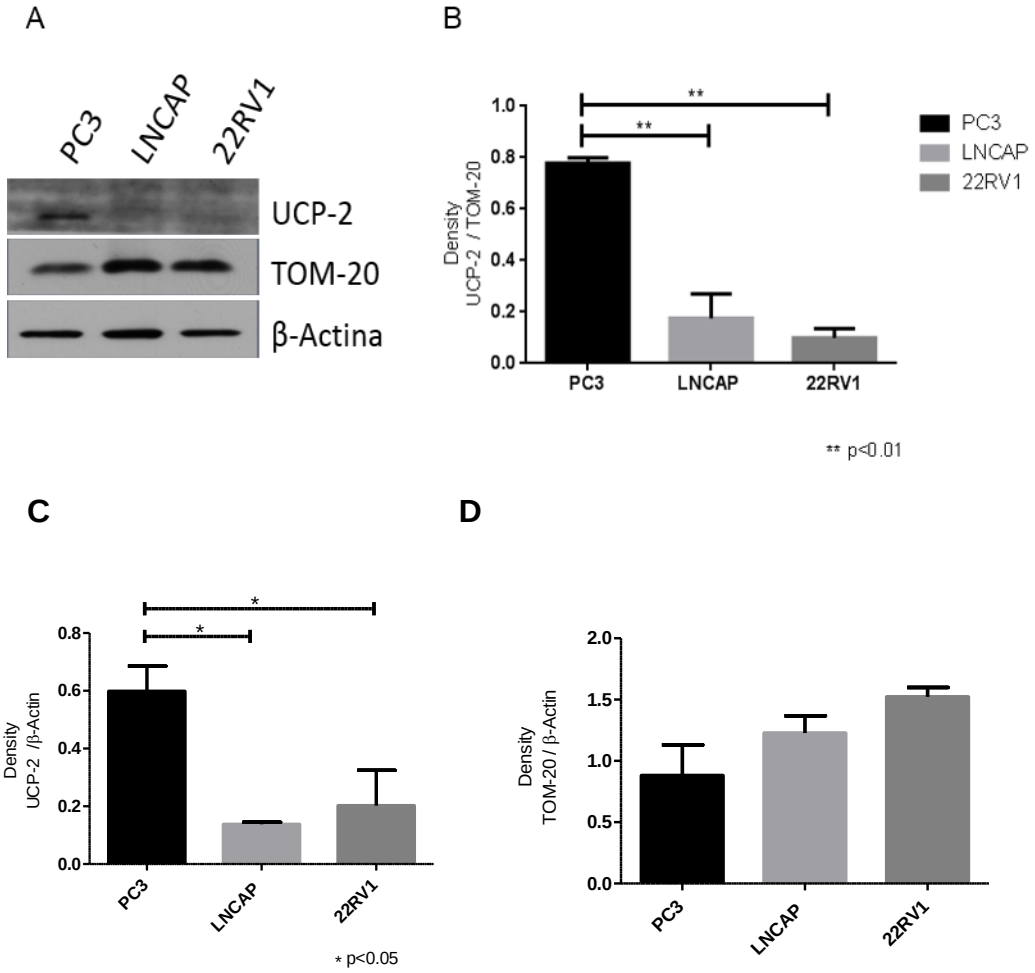


Figure 8. Analysis of UCP-2 protein expression. A) Western blot analysis for the UCP-2 protein, β-Actin as an exogenous control and TOM-20 as a control of mitochondrial loading. A representative immunoblot of two experiments is shown. B) Quantification of UCP-2 protein in the cell lines, expressed as density of UCP-2 band per density of TOM-20. C) Quantification of UCP-2 protein in the cell lines, expressed as density of UCP-2 band per the density of the β-Actin. D) Quantification of TOM-20 protein in the cell lines, expressed as density of TOM-20 band per density of β-Actin. Bars, standard deviation; columns, mean. The asterisks indicates the level of statistical significance (**p<0.01). Data were subjected to one-way ANOVA and a posterior Tukey test.

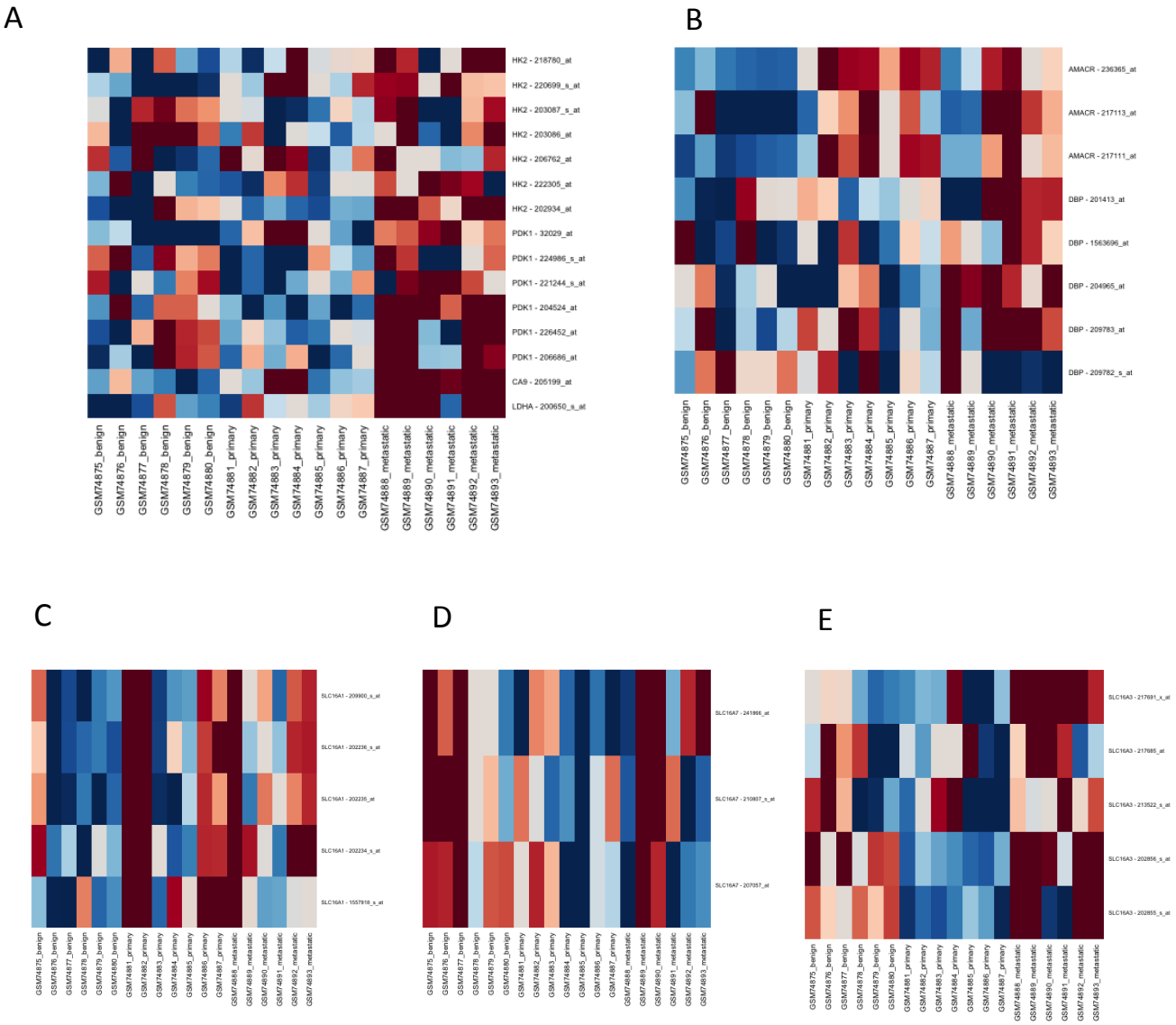


Figure 9. Proteins involved in glycolytic and fatty acid oxidation pathway were also found to be aberrantly expressed in prostate cancer specimens. Heatmap showing mRNA levels of HK2, PDK1, CA9 and LDHA. AMACR, DBP and ACOX3 are also represented in the Varambally dataset (Figure 9A and 9B). Red: high; blue: low. Heatmap was generated using a galaxy-based CRI Bioinformatics Core Facility tool. **SLC16A3 is aberrantly expressed in metastatic prostate cancer specimens.** Heatmap showing mRNA levels of SLC16A1 (MCT1) (Figure 9C) , SLC16A7 (MCT2) (Figure 9D) and SLC16A3 (MCT4) (Figure 9E) in the Varambally dataset.

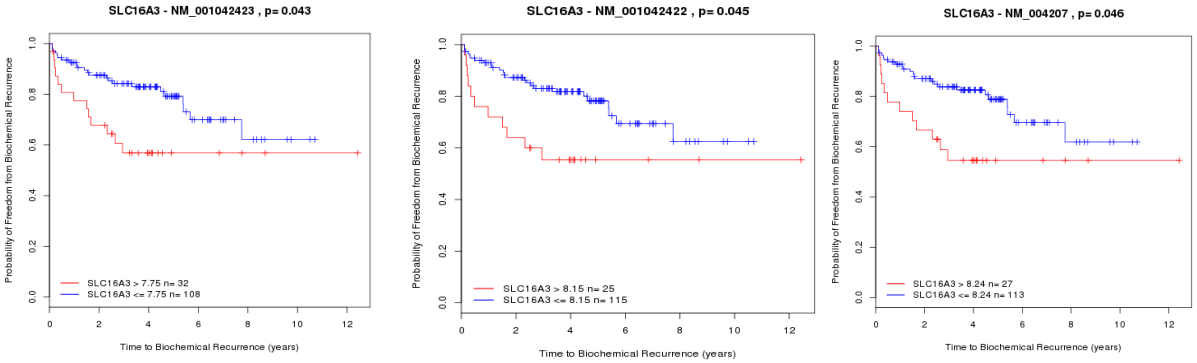


Figure 10. Kaplan-Meyer plots for SLC16A3 based on Glinsky et al., 2004. Recursive partitioning was performed using a galaxy-based CRI Bioinformatics Core Facility tool.

4.3.2 Exploitation of MCTs as potential therapeutic targets in prostate cancer .

Abstract

The metabolic phenotype of many tumours switches from oxidative phosphorylation (used by the majority of normal epithelial cells) to aerobic (Warburg effect) or anaerobic glycolysis. This phenomenon significantly increases the rates of glucose consumption and lactate production, enabling tumours to meet their energy and biosynthetic demands even under conditions of low nutrients and oxygen. However the end-product of glycolysis, lactate, is a metabolic “dead end”, which if allowed to accumulate in the tumour cell can cause feedback inhibition of glycolysis, intra-cellular acidification and inhibition of cell growth. Therefore, pharmacological inhibition or disruption of lactate metabolism is a promising therapeutic strategy to target a range of human cancers. In this study we evaluate the effect of the compound CHC in different in vitro models of prostate cancer progression and aggressiveness and also we test the effect of specific inhibition of each MCT isoform using siRNA.

We show that selective inhibition of lactate transport by MCT1 and MCT4 inhibition decreases the viability and proliferation of prostate cancer cell lines in normoxic and hypoxic conditions and might offer a novel mechanism for targeting the metabolic phenotype of tumours. However further studies need to be done in order to assess the effect of MCTs inhibition in the metabolism of prostate tumour cells.

Introduction

Although prostate cancer (PCa) is one of the most incident cancers worldwide in men, there is a lack in terms of effective therapies against this disease being the existent ones invasive for patients. Thus, studies exploring novel therapies are urgently needed.

Our previous reports are in accordance with some papers in the literature that describes prostate cancer as a tumour that does not rely mainly on glycolytic metabolism but instead glycolytic metabolism is mainly found in the advanced stages of the disease [1,2]. As so, understanding the metabolism of these tumours can identify new molecular targets with clinical relevance.

If MCTs can represent suitable targets in the metabolism of CaP is not well elucidated. An in vivo study from Kim and colleagues showed that MCT1 inhibition did not have a significant effect on tumour volume, although it was associated with increased necrotic fraction [3]. Since there are no reports yet about the true relevance of MCTs inhibition in the development and progression of PCa, it is important to invest in studies evaluating both the blocking and silencing of MCTs in this tumour.

We aimed to evaluate the effect of MCT inhibition in PCa cell lines using CHC, a classical MCT1 inhibitor and also siRNAs to target different MCTs isoforms in different models of disease progression.

Materials and Methods

To understand the functional role of MCTs in tumor cells, we will evaluate the effect of MCT activity inhibition on tumor cell viability and proliferation in different prostate tumour cell lines. Activity inhibition studies were undertaken, using the MCT classical inhibitors, such as CHC, or RNAi specific for each MCT isoform.

Cell Lines and Cell Culture

Human prostate cell lines representing different relevant features of prostatic adenocarcinoma were used. The selected cells lines were obtained from ATCC-

American Type Culture Collection, MD, USA. Cell lines were grown in RPMI-1640 cell culture medium supplemented with 10% Fetal Bovine Serum (FBS) (GIBCO®, Invitrogen,Carlsbad, CA) and 1% Penicillin-Streptomycin (P-S) (GIBCO®, Invitrogen, Carlsbad, CA).

Downregulation of MCTs expression

Silencing of MCT1, MCT2 and MCT4 expression was performed using siRNA from Qiagen. Final siRNA concentration used was 10nM. Lipofectamine RNAiMax (Invitrogen) was used as permeabilization agent, according to the manufacturer`s instructions.

Drugs

Alpha-cyano-4-hidroxycinnamate (CHC Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO Sigma-Aldrich) to perform the stock and working solutions.

Cell Viability and Proliferation Assays

Different techniques were used to study the effect of MCTs inhibition in prostate cell lines.

To study the effect of CHC in prostate cells, the cells were plated into 96-well plates and the effect of CHC treatment was determined at 24, 48 and 72h by sulforhadamide B assay (SRB, TOX-6;Sigma-Aldrich), according to the manufacturer`s recommendations. IC50 values (i.e., CHC concentration that corresponds to 50% of cell growth inhibition) were estimated from 3 independent experiments, each one in triplicate using GraphPad Software.

To study cell proliferation and viability, after treatment with siRNA cells were seeded in twelve-well plates. Reverse transfection was carried out on the day of seeding; to assess the effects of drug treatments, cells were allowed to attaché for 24 hours before treatment. Three and Six days after seeding, cells were harvested by trypsinization. In order to quantify both living and dead cells, the culture medium was saved and combined with the harvested cells. Cells were pelleted by centrifugation and resuspended in 500 ul PBS. Cell number and viability were determined using Beckman Coulter Vi-Cell. Duplicate samples were prepared for each experimental condition. Also using the Incucyte equipment growth curves

were obtained for different prostate cancer cell lines during one week with siRNA transfection at day 2.

MTS assay was also used in a large variety of cell lines as a fast method to screen the effects of MCTs inhibition in cells viability.

Metabolism Assay (Extracellular Glucose and Lactate Measurements)

Cells were plated in 48-well plates and allowed to adhere overnight. Then cells were treated with 5 mM of CHC and the cell culture medium was collected after 8, 12 and 24h for glucose and lactate quantification. Glucose and lactate were quantified using commercial kits (Roche and Spinreact, respectively), according to the manufacturer`s protocols.

Results

The use of CHC does not affect the lactate production in prostate cell lines.

The MCT inhibitor (CHC) decreased the total cell biomass of PNT2 and PC3 cells, while LNCaP was less sensitive along time (Figure 1). IC_{50} values were slightly higher in LNCaP than PC3 and PNT2 along time. The effect of CHC on prostate cell metabolism was evaluated using the IC_{50} values obtained by SRB assay at 48 hours. MCT inhibition does not induce a decrease in lactate production in prostate cell lines (Figure 2).

MCTs 1, 2 and 4 specific inhibition differentially affect prostate cell lines under normoxic conditions

The effect of the specific inhibition of different MCT isoforms, using siRNA, in the viability of prostate cell lines is shown in Figure 3. MTS results showed a significant decrease on prostate cell lines viability when MCT1 is inhibited. Regarding MCT2, its inhibition seems to affect namely PNT1a and PC3 cell lines whereas MCT4 inhibition affects PNT1a, C4-2 and PC3 cells viability. Curiously, LNCaP which was previously observed to be the less glycolytic of all the prostate cancer models was the only cell line that did not show any sensitivity to MCTs inhibition. In order to confirm the results obtained by MTS, vi-Cell counter

equipment was also used to assess the viability of cells after MCTs inhibition (Figure 4). This experiment was so far performed on PNT1a, LNCaP and C4-2B cells.

The results obtained using vi-cell counter were in accordance to the ones obtained by MTS. PNT1a and C4-2B showed a decreased on viability when exposed to MCT1 and MCT4 inhibitors. Again, LNCaP showed no evident alterations in their viability when the different MCT isoforms were inhibited.

MCTs 1, 2 and 4 specific inhibition differentially affect prostate cell lines under hypoxic conditions

Due to the already mentioned importance of hypoxia with subsequent activation of HIF and its implications in tumour metabolism plus the early observation that MCT4 mRNA levels clearly increased in hypoxic conditions, we assessed whereas cells under hypoxic conditions will be more dependent on these transporters and so more susceptible to their inhibition. Figure 5 shows the effect of MCTs inhibition under hypoxic conditions in cell lines viability measured by MTS.

Accordingly, in the results obtained by MTS assay an evident decrease in cell viability was shown in PNT1a and C4-2B when MCT1 and MCT4 are inhibited. Again, a decrease on LNCAPs viability with MCT1 inhibition under hypoxic conditions was observed (Figure 6)

MCT1 inhibition affects the proliferation of prostate cell lines under normoxia

In order to assess the effects of MCTs inhibition on the proliferation of prostate cancer cells, three different models of prostate cancer cell lines were used and the growth curves were obtained using the Incucyte equipment. The cells were incubated for one week and transfected at day two. Figure 7 shows the growth curves for LNCaP, C4-2 and C4-2B cell lines before and after MCTs inhibition. The arrow indicates the day in which cells were transfected.

The cell models studied showed a decrease in cell proliferation after MCT1 inhibition. Importantly, while previously LNCaPs showed no decrease on cell

viability upon MCTs inhibitor under normoxic conditions, a decrease in cell proliferation was observed when MCT1 was inhibited in this cell line.

Discussion

The increase in lactate production as a consequence of the high levels of glycolytic metabolism by tumour cells even in normoxic conditions is widely described in several types of cancer and monocarboxylate transporters have been described as upregulated in several tumours as a consequence of this change in cells metabolism. This fact makes MCTs attractive targets for cancer therapy, however, the knowledge on MCTs as potential targets in prostate cancer therapy stills scarce.

We observed that MCTs inhibition using CHC appears not to affect glycolytic metabolism of prostate cells, supporting the lower dependence of prostate cancer on glycolytic metabolism. Importantly, PNT1a and PC3 were the cell lines that showed higher sensitivity to CHC inhibition although this inhibition did not affected glucose consumption or lactate production. Being CHC a compound with much higher affinity for MCT1 than MCT4 these results are not surprising since our theory supports a lactate efflux through MCT4 in PC3 cells and not MCT1. Also the fact that the non-malignant cell line PNT1a showed higher levels of glicolytic metabolism and sensitivity to CHC might be explained by the fact that this cell line has a truncated Krebs cycle, relying less on OXPHOS than LNCaPs. Curiously, LNCaPs showed an increase in glucose consumption when MCT1 was inhibited. In theory we believe that MCT1 is being use for lactate uptake and so, the inhibition of MCT1 by CHC will lead to an increase demand of glucose to feed Krebs cycle since lactate uptake as a fuel was being blocked by the use of this drug. We have already some evidence that LNCaPs are able to consume lactate (data not shown), but further studies are being developed in order to prove this theory.

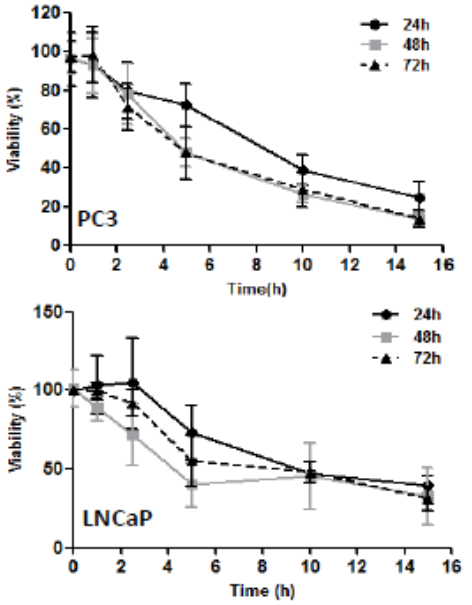
Using siRNA to specifically inhibit different isoforms of MCTs we found out that the inhibition of different isoforms in different cell lines and under different environment conditions affect in different ways prostate cells. This complexity corroborates our previous hypothesis indicating that MCTs are activated differentky across prostate malignant transformation and so these results are complex and need to be interpreted considering the metabolic heterogeneity of prostate cancer disease.

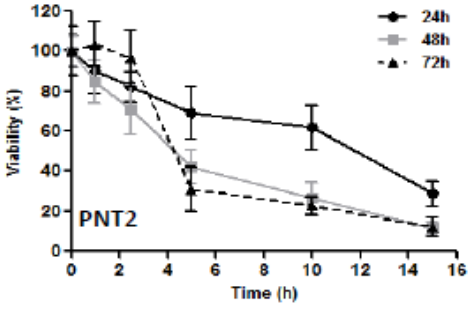
However, in a general way we can say that even under normoxia or hypoxia MCT1 seems to be a suitable target for prostate cancer cells, whereas MCT4 inhibition effect on cells viability and proliferation in more evident under hypoxic conditions. More studies are being developed in order to characterize the effect of MCTs inhibition accordingly to different phases of disease progression.

References

1. Zha, S., et al., *Peroxisomal branched chain fatty acid beta-oxidation pathway is upregulated in prostate cancer.* Prostate, 2005. **63**(4): p. 316-23.
2. Liu, Y., L.S. Zuckier, and N.V. Ghesani, *Dominant uptake of fatty acid over glucose by prostate cells: a potential new diagnostic and therapeutic approach.* Anticancer Res, 2010. **30**(2): p. 369-74.
3. Kim, H.S., et al., *Carbohydrate restriction and lactate transporter inhibition in a mouse xenograft model of human prostate cancer.* BJU Int, 2012. **110**(7): p. 1062-9.

Figures





IC_{50} (mM)	24h	48h	72h
PC3	7.74	5.05	5.02
PNT2	10.09	4.24	4.45
LNCaP	10.81	5.98	6.69

Figure 1. Effect of CHC on total cell biomass of prostate cell lines along time (sulphorhodamine B assay).

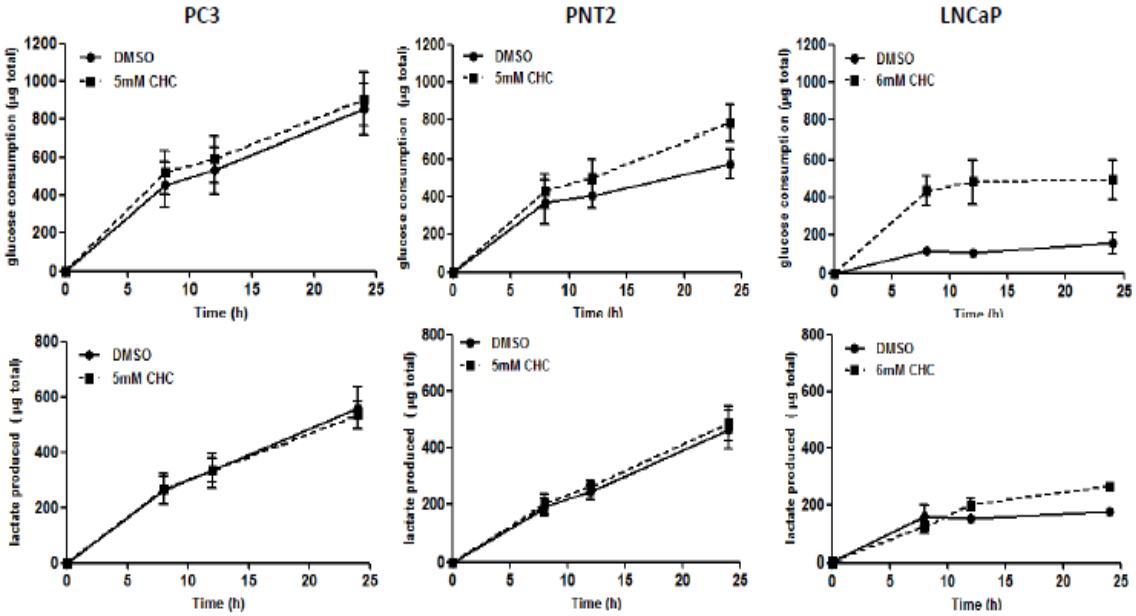


Figure 2. Effect of CHC on glucose consumption and lactate production, along time, in prostate cancer cell lines.

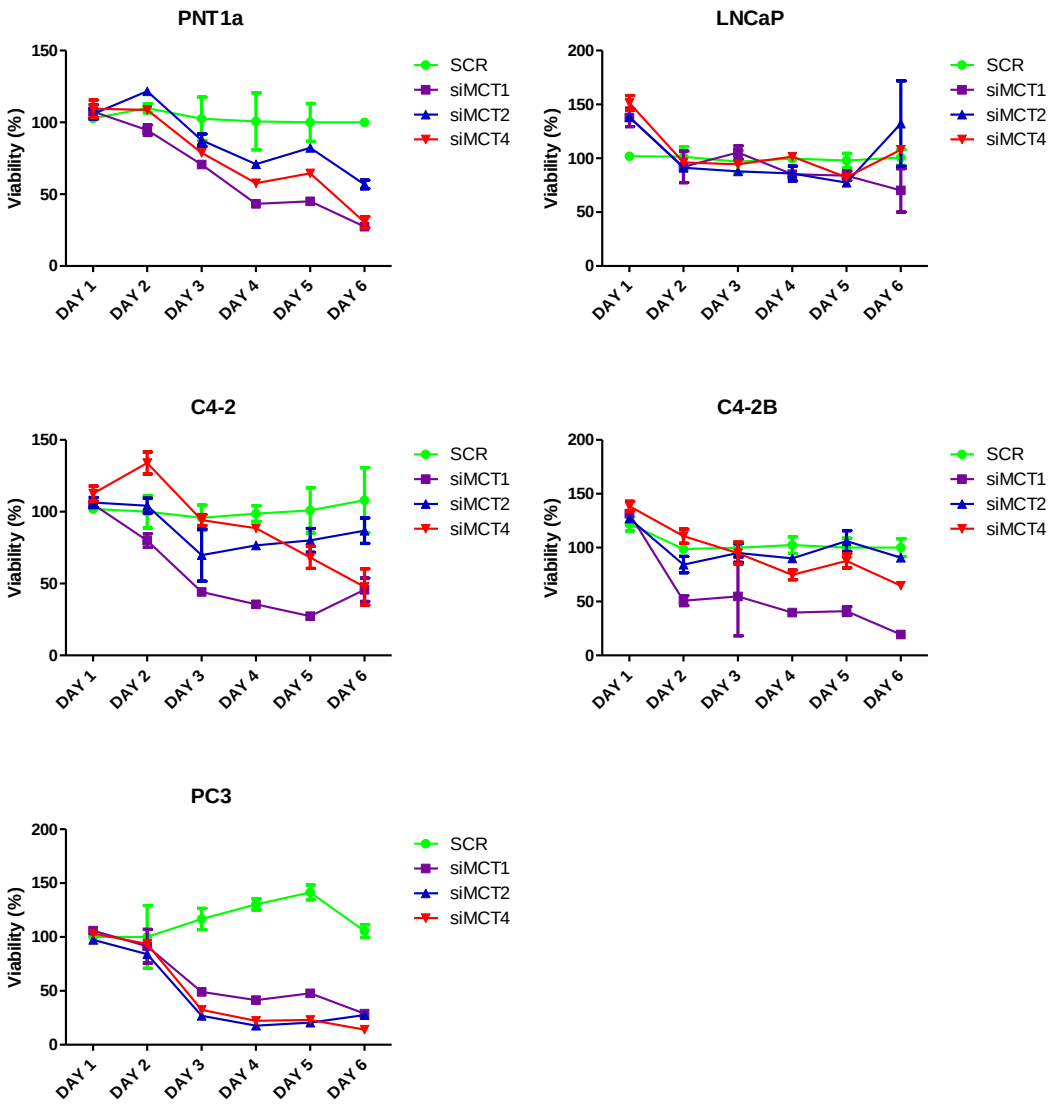


Figure 3. Effect of MCTs inhibition on cell viability measured by MTS assay on PNT1a, LNCaP, C4-2, C4-2B and PC3 cell lines.

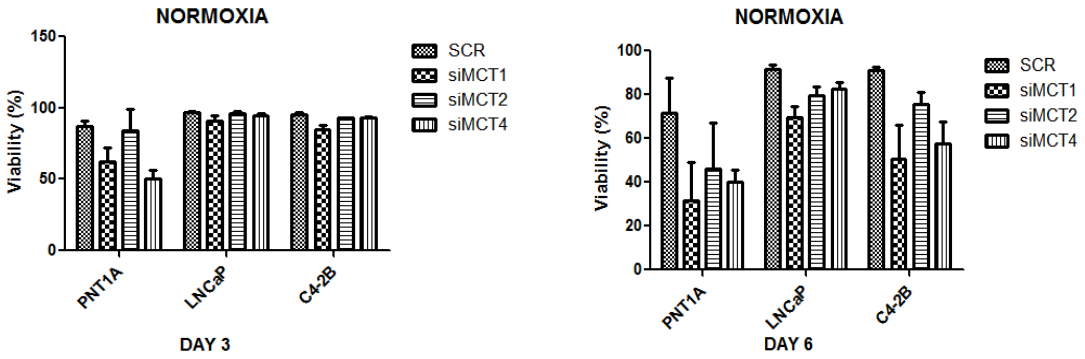


Figure 4. Percentage of viable cells after treatment with MCTs inhibitors on Day 3 and Day 6.

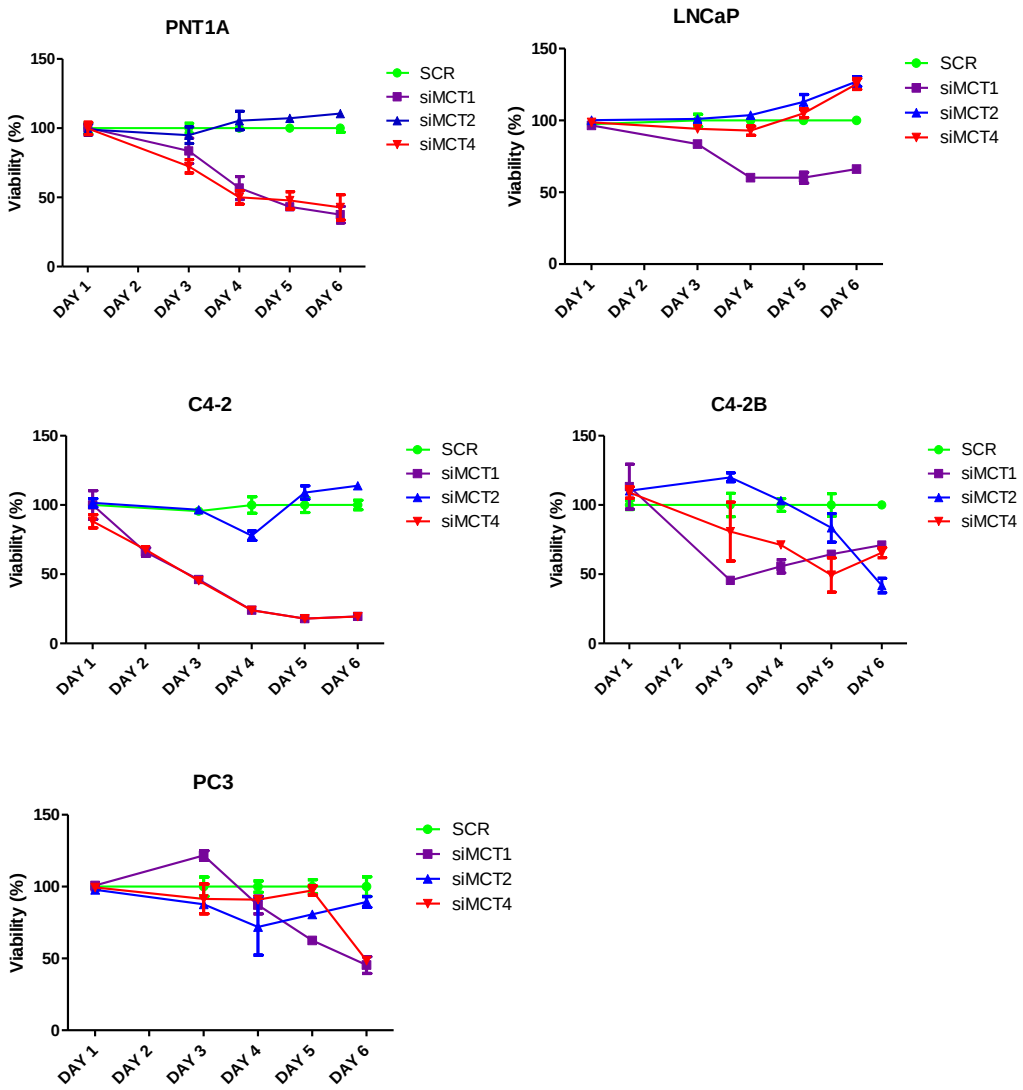


Figure 5. Effect of MCTs downregulation on cell viability under hypoxic conditions measured by MTS assay on PNT1a, LNCaP, C4-2, C4-2B and PC3 cell lines.

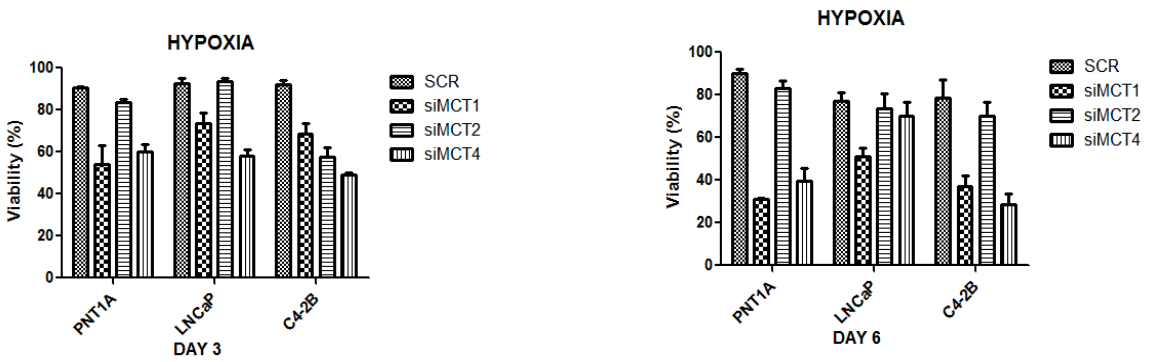


Figure 6. Percentage of viable cells after downregulation of MCTs on Day 3 and Day 6.

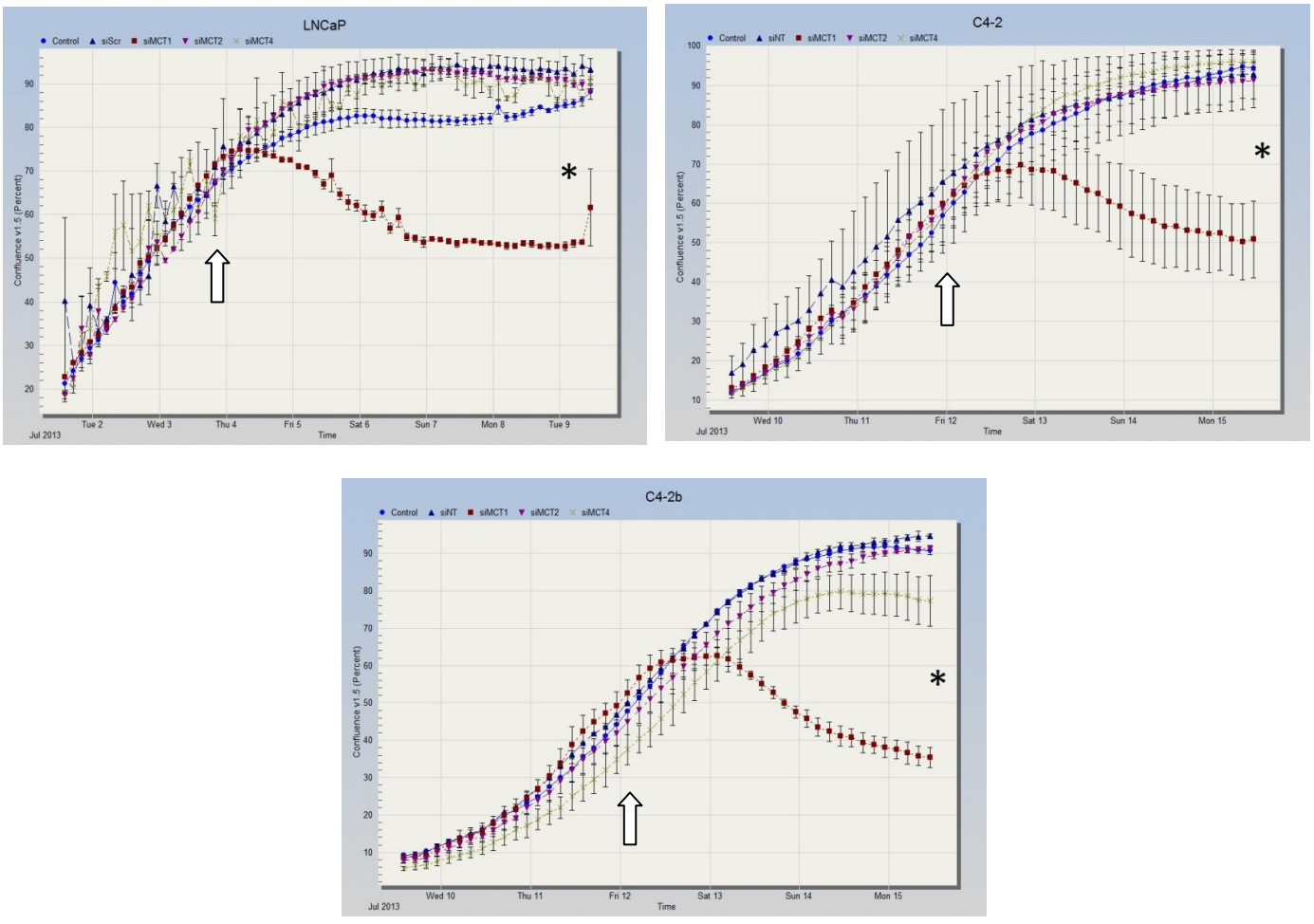


Figure 7. Growth curves obtained using Incucyte equipment to assess the growth of cancer cells, namely LNCaP, C4-2 and C4-2B before and after MCTs inhibition.

CHAPTER 5. A ROLE FOR MCTs IN PROSTATE CANCER ENVIRONMENT

5.1 CHAPTER OVERVIEW

It is well established that solid tumours, including prostate cancer, exist under fluctuating oxygen tensions and are exposed to hypoxia. Tumour cells under hypoxia primarily use glucose for glycolytic energy production producing lactate which is expelled to the tumour microenvironment, allowing tumours to continue their glycolytic activity. Recent works postulated that lactate which was generally considered a waste product is preferred by tumour cells over glucose as primary energy source. If so, this aspect creates the perfect conditions for a symbiosis between anaerobic glycolytic cells and aerobic cells from the environment or vice versa.

In the previous chapters we showed that prostate cancer cells do not rely primarily in glycolysis, but the metabolic switch to a high glycolytic phenotype seems to occur across malignant progression. In this perspective, we aimed to also evaluate the possible changes in the fibroblasts across malignant transformation and also if the theory on the “Reverse Warburg effect” could be suggested in prostate cancer model.

In this chapter, we present results submitted for publication where we infer about interactions between CAFs and prostate tumour cells, analyzing the expression of key metabolic-related proteins in CAFs in relation to prostate cancer using a large series of prostate samples and assessing the clinico-pathological significance of this expression in order to infer a possible CAFs signature for PCa progression.

We observed in CAFs a highly expression of MCT4 and CAIX corroborating the hypothesis of a “Reverse Warburg effect” in prostate cancer in which fibroblasts are under oxidative stress expressing CAIX, one of the best cellular biomarkers of hypoxia. Interestingly, we observed that alterations in metabolic-related proteins expression are already evident across malignant transformation and for the first time we observed that cases which show high MCT4 expression in CAFs with concomitantly strong MCT1 expression in PCa cells are associated with poor clinical outcome, namely presence of biochemical recurrence after surgery.

5.2 PUBLISHED RESULTS

The results presented in this chapter were:

(i) Submitted for publication as an original article in an international peer reviewed journal

Pértega-Gomes N, Vizcaíno JR,Gouveia C, Lopes C. and Baltazar F. "A lactate shuttle system between tumour and stromal cells is associated with poor prognosis in prostate cancer". 2013.

(ii) Presented as poster in the following international scientific meeting:

Pértega-Gomes N. Vizcaíno JR, Lopes C. and Baltazar F. "Comparison of Metabolic Pathways between cancer and stromal cells in prostate carcinoma. A role for Monocarboxylate transporters (MCTs) in tumour-stroma cross-talk." at FEBS Advanced Lecture Course on Translational Cancer Research, Algarve, Portugal. 2011.

(iii) Presented as poster in the following national scientific meeting:

Pértega-Gomes N. Vizcaíno JR, Lopes C. and Baltazar F. "Monocarboxylate transporters 1 and 4 are upregulated in stroma cells surrounding malignant glands and PIN lesions" at Congress of Portuguese Society of Pathology, Porto, Portugal. 2011.

5.3.1 A Lactate shuttle system between cancer and stromal cells mediated by Monocarboxylate transporters (MCTs) is associated with poor prognosis in prostate cancer

A lactate shuttle system between tumour and stromal cells is associated with poor prognosis in prostate cancer

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Abstract

Objectives - Cancer cells are embedded in stromal cells, namely cancer associated fibroblasts (CAFs) that are now accepted as important players in cancer dynamics, being involved in the tumour growth and progression. Although various reports about this interaction exist, the clinico-pathological significance of this cross-talk is still largely unknown. In this study we aimed to characterize the expression of key metabolic proteins involved in the glucose transport, pyruvate/lactate shuttle system, glycolytic metabolism and fatty acid oxidation, in stroma (fibroblasts) across malignant transformation and differences between cancer cells and CAFs, assessing the clinico-pathological significance of this expression.

Materials & Methods – Patients in the study were men who performed radical prostatectomy for prostate cancer treatment without any prior hormonal therapy. Prostate tissues were obtained from 480 patients with a median age of 64 years, who performed radical prostatectomy. Tissues were analysed for the expression of several key metabolic related proteins in glands and surrounding fibroblasts by immunohistochemistry. Reliable markers of prognosis such as, pT stage and biochemical recurrence were assessed for each case.

Results - We observed that prostate cancer cells do not seem to rely mainly on glycolytic metabolism, while we observed a high expression of MCT4 and CAIX in CAFs, suggesting that fibroblasts are under oxidative stress expressing CAIX, one of the best cellular biomarkers of hypoxia. Curiously, we observed that alterations in the expression of metabolism-related proteins are already evident across malignant transformation, suggesting a progressive alteration of CAFs from an

early stage. Additionally, and for the first time, we observed that cases that show high MCT4 expression in CAFs with concomitantly strong MCT1 expression in prostate cancer (PCa) cells are associated with poor clinical outcome, namely presence of biochemical recurrence after surgery and pT3 stage of the tumour.

Conclusions - In sum, this work shows for the first time a clinico-pathological value for the lactate shuttle in prostate cancer, raising the possibility to explore further alterations in CAFs as prognostic factors and further support the use of MCT1/MCT4 as targets for PCa therapy.

Introduction

It is well established that solid tumours, including prostate cancer, exist under fluctuating oxygen tension, being exposed to hypoxia [1,2]. Tumour cells under hypoxia use primarily glycolysis for energy, producing lactate, which is expelled to the tumour microenvironment, allowing tumours to continue their glycolytic activity [3,4]. Recently, Sonveaux *et al.* showed that lactate, which is generally considered a waste product, is preferred by oxidative tumour cells over glucose, as primary energy source [5]. If so, this aspect creates the perfect conditions for a symbiosis between anaerobic glycolytic cells and aerobic cells.

Monocarboxylate transporters (MCTs) have been identified by our group to play an important role in various tumours [6] however, since they facilitate the transport of lactate in and out of the cells, their role in this stromal/epithelial cells symbiosis is also attracting interest. MCT1 is a high-affinity transporter and its expression seems to be regulated by multiple signaling pathways, microenvironmental parameters, changes in substrate concentration and pH. MCT4 is a low affinity transporter, which is abundantly present in highly glycolytic muscle cells and is also one of the many target genes of hypoxia-inducible factor 1 alpha (HIF- 1α) [7]. Other targets of HIF-1α include glucose transporter-1 (GLUT-1), the main transporter involved in glucose uptake [8], lactate dehydrogenase V (LDHV), responsible for the conversion of pyruvate into lactate, pyruvate dehydrogenase kinase isozyme 1 (PDK1) that is responsible for the phosphorylation and consequently inactivation of pyruvate dehydrogenase PDH that contributes to transforming pyruvate into acetyl-CoA [9] and carbonic anhydrase IX (CAIX), a hypoxia-related protein involved in pH regulation [10]. We also aimed to evaluate alpha-methylacyl-CoA racemase (AMACR), pristanoyl - CoA oxidase (ACOX-3) and D-bifunctional protein (DBP) expressions since increasing data point to the importance of these fatty acid oxidation related proteins in prostate cancer [11,12].

In this study, we aimed to infer about possible metabolic interactions between CAFs and prostate cancer cells, by analyzing the expression of key metabolic-related proteins in CAFs in relation to prostate cancer using a large series of

prostate samples. We also assessed the clinico-pathological significance of this expression to infer a possible CAFs signature for PCa progression.

Materials and Methods

Patient sample selection

Prostate cancer patients were selected according to the following criteria: availability of both tumour and normal tissue for each patient; presence of adequate amount of stroma in both normal and tumour tissues for efficient selection for tissue microarray construction (TMA); and absence of chemotherapy or radiotherapy. Prior to TMA construction, tissue morphology was assessed on HE slides. Formalin–fixed paraffin embedded tissues from 480 prostate cancer patients were retrieved from the archives of the Department of Pathology of Centro Hospitalar do Porto, Portugal. Stroma surrounding non-neoplastic glands, PIN lesions and malignant glands was also analyzed.

Ethics

The work has been approved by DEFI (Departamento de Ensino Formação e Investigação) Ethics Committee of Centro Hospitalar do Porto ref. no. 017/08(010-DEFI/015-CES).

Immunohistochemistry

Samples organized into TMAs including 203 non-neoplastic, 176 PIN and 480 neoplastic tissues were analyzed for MCT 1 and 4, GLUT-1, GLUT-12, LDHV, PDK1, CAIX, AMACR, ACOX-3 and DBP expressions. Staining was evaluated using a combined score system, as previously described [13].

Detailed information regarding immunohistochemistry technique is given in **Table 1**.

Immunohistochemical evaluation

IHC evaluation was performed evaluating the intensity and the extension of the staining, as previously described [13] and blindly by two independent observers, who were blind to the clinico-pathological data of the patients.

Immunoreaction in TMA sections was evaluated for cytoplasmic and/or plasma membrane staining. Shortly, sections were scored semi-quantitatively as follows: 0: 0% of immunoreactive cells; 1: < 5% of immunoreactive cells; 2: 5-50% of

immunoreactive cells and 3: > 50% of immunoreactive cells. Also, intensity of staining was scored semi-quantitatively as follows: 0: negative; 1: weak; 2: moderate and 3: strong. The final score was defined as the sum of both parameters (extension and intensity), and grouped as negative (scores 0-3) and positive (scores 4-6). Discordant results in different cores of the same case were scored as follows: average of extension plus highest intensity score. Discordant results were discussed in a double-head microscope. For statistical purposes, only moderate and strong immunoreaction final scores were considered positive. Discordant cases were discussed in a double-head microscope in order to determine a final score.

Statistics

Statistical analysis was performed using the SPSS statistical software (version 17.0, SPSS Inc., Chicago, IL, USA). All comparisons were examined for statistical significance using Pearson's chisquare (χ^2) test, being the threshold for significance $p<0.05$.

Results

Figure 1 shows the percentage of positive cases for each of the key metabolic-related proteins studied in cancer-associated fibroblasts (CAFs) comparing to epithelial cells of malignant glands. We observed evident differences between the expression of these proteins in fibroblasts surrounding tumour and cancer cells. MCT1, MCT4, LDHV, PDK1, GLUT-1, GLUT-12, CAIX, AMACR, ACOX-3 and DBP are differently expressed between CAFs and tumour epithelial cells, while MCT1, LDHV, GLUT-1, GLUT-12, AMACR, ACOX-3 and DBP were exclusively expressed in prostate cancer cells. MCT4 and CAIX are more expressed in CAFs and PDK1 stained both malignant glands and CAFs.

Additionally, we studied if fibroblasts exhibited differences in protein expression across malignant transformation by analyzing the expression of the same proteins in fibroblasts surrounding benign glands (BAFs), PIN lesions (PAFs) and finally cancer associated fibroblasts (CAFs).

Figure 2 shows the heatmap representing the expression of MCT4, PDK1 and CAIX. The samples are divided in three groups: BAFs, PAFs and CAFs. The groups are red-colour coded to the left of the heatmap, and protein names are given below each column. For visualization purposes the expressions have been scaled between 0 and 3,accordingly to the final score of the staining for each case, as so, no staining (0), weak staining (1), moderate (2) and strong (3) staining are represented.

All proteins that exhibited expression in CAFs are also present in some cases surrounding precursor lesions but not so evident surrounding benign glands. This is the case for MCT4 and PDK1, since CAIX curiously was also observed in fibroblasts surrounding non-neoplastic glands: benign glands and PIN lesion.

Figure 3 shows representative immunohistochemical staining of the key metabolic-related proteins in fibroblasts and prostate glands across malignant transformation, i.e. from fibroblasts surrounding non-neoplastic glands to fibroblasts surrounding PIN lesions and finally cancer associated fibroblasts. It is possible to observe an evident expression of MCT1 in the plasma membrane of prostate glands with no expression in the surrounding stroma. In contrast, we can see the increased expression of MCT4 in fibroblasts across malignant transformation with no staining in the prostate glands. PDK1 expression was detected in both glands and stroma, whereas CAIX was only detected in stroma with no staining in prostate glands.

Associations between expressions of the metabolic proteins and clinico-pathological data are presented in Table 2. Preoperative serum total PSA, clinical stage, perineural invasion and biochemical recurrence were selected for this study since these are some of the most important variables found to influence outcome. We observed that CAIX expression was associated with presence of biochemical recurrence after surgery ($p=0.003$) when expressed in CAFs and PDK1 showed a strong tendency to correlate with the presence of perineural invasion ($p=0.069$). Curiously, we found that the most interesting associations with the clinico-pathological parameters were observed by considering the tumours that express MCT1 in tumour cells with concomitant expression of MCT4 in the surrounding CAFs . These cases were associated with the presence of biochemical recurrence

after surgery (p= 0.052) and pT3 stage (p=0.009) of the tumour. Additionally, cases negative for both or only with MCT1 present in the malignant glands, with no MCT4 in CAFs, showed no associations with clinico-pathological parameters (data not shown). Figure 4 shows a schematic representation of the the main findings regarding protein expression from radical prostatectomy specimens. Again, the lactate shuttle between CAFs and PCa cells that we found to exhibit clinico-pathological relevance.

Discussion

Several research groups have recently focused their interest on the role of cancer associated fibroblasts in the progression and metastization of prostate cancer, showing that a dynamic interaction between stroma and epithelium might play a critical role in this progression [14-17]. Thus, the critical role played by the cross-talk between stroma and epithelium in carcinogenesis and prostate cancer progression has been increasingly recognized. In this work we provide evidence for the possible interactions between cancer cells and the surrounding fibroblasts in terms of metabolic cooperation, examining the expression of major proteins involved in cellular metabolism, focusing on differences between cancer cells and tumour-associated fibroblasts as well as in fibroblasts across malignant transformation and the possible clinico-pathological significance of this expression. In this study, an effort in order to categorize the protein expression of stromal cells associated with prostate cancer was made to categorize a compartment that is not well studied and will contribute to an improved understanding of prostate cancer. We firstly observed that there were significant differences between CAFs and tumour glands relative to the expression of key metabolic proteins. In particular, CAIX and MCT4 selectively label cancer associated fibroblasts in contrast to malignant glands where CAIX and MCT4 were only present in very few cases. On other hand, a distinct clear and strong membranous expression of MCT1 was noted consistently in cancer cells, suggesting a role in the transport of lactate in tumor cells from the acidic extracellular matrix, possibly meaning that lactate may be used as a fuel by oxidative cancer cells. Also, we observed that proteins involved in fatty acid oxidation are restricted to the tumour cells, which is compatible with the existence of a metabolic pathway different from glycolysis but compatible with OXPHOS in prostate cancer cells.

The expression levels of GLUT1, a key transporter in the uptake of glucose by cells, are expected to define the rates of glucose influx into the cells. In the present study, CAFs did not show GLUT1 or GLUT-12 expression, but this presumably reflects the limits of immunohistochemical technique to detect membranous GLUT protein at the baseline concentrations present in CAFs. Similarly, as previously observed by us in prostate cancer cells, only very few cases were positive for

GLUT-1 and GLUT-12 and this expression was not even present at the plasma membrane, suggesting a low level of activity of these proteins. Thus, assessment of other GLUT isoforms may be worth. Also, looking for other proteins already explored by us in cancer cells we found that also PDK1 expression, although not overexpressed in cancer associated fibroblasts, exhibited a tendency to correlate with the presence of perineural invasion which is considered a useful prognostic factor for predicting extra-prostatic extension and the involvement of surgical margin in the radical prostatectomy specimen.

Interestingly, we also observed that protein expression in prostate fibroblasts changes across malignant transformation, suggesting that also the already existing stroma might suffer alterations and plays a role in this metabolic adaptation of cancer cells beyond the well studied role of newly formed stroma.

From the above immunohistochemical findings, it seems that a contribution to overcoming the adverse microenvironment in prostate cancer may derive from well-organized metabolic domains composed of tumor cells and CAFs. Our hypothesis is in agreement with the results of Fiaschi *et al* [18] that describe the metabolic reprogramming of CAFs towards the Warburg phenotype as a result of contact with prostate cancer cells. Using *in vitro* studies, they showed lactate production and extrusion by *de novo* expressed MCT4 in CAFs and also that prostate cancer cells upon contact with CAFs, were reprogrammed towards aerobic metabolism, with an increase in lactate upload via the lactate transporter MCT1. In agreement, pharmacological inhibition of MCT1-mediated lactate upload dramatically affected PCa cell survival and tumor outgrowth, however in this study they do not show any data regarding clinico-pathological associations and few cases were assessed. Another study by Giatromanolaki *et al* [19] suggests the opposite, also based on immunohistochemical findings, they described an energy recycling path between the aerobic stroma and the anaerobic cancer cells within the framework of the Warburg effect. Their conclusions are mainly based on the observation that LDH1 is evidently expressed in CAFs and the presence of MCT1 that was also found mainly in prostate cancer cells was justified with a role in lactate extrusion and not in the uptake, as we believe it is. We recognize the importance of assessing LDH1, however, in the present study we assessed for the first time MCT4 and CAIX as important markers of hypoxia in a larger series. Also,

we assessed important clinico-pathological parameters and we found associations with poor prognosis, raising once more the possible role of CAFs in disease management. We believe that these changes could be, and probably are more likely to be a byproduct of tumour biology with further influence on patients outcomes that need to be deeper explored.

In summary, we examined cancer associated fibroblasts and differences between cancer cells and CAFs using tissues from 480 patients, showing the high expression of MCT4 and CAIX in CAFs and showing for the first time that there is a clinico-pathological significance for the MCT1 expression in tumour cells with concomitantly MCT4 expression in the fibroblasts of the same tumours.. In fact, it seems that the stromal expression of hypoxia regulated proteins is an adverse prognostic factor in prostate carcinomas, suggesting that tumor hypoxia may influence tumor-associated stromal cells in a way that ultimately contributes to patient prognosis.

References

1.Stewart GD, Ross JA, McLaren DB, Parker CC, Habib FK, Riddick AC. **The relevance of a hypoxic tumour microenvironment in prostate cancer**. BJU international. 2010;105(1):8-13. Epub 2009/11/06.

2.Movsas B, Chapman JD, Hanlon AL, Horwitz EM, Pinover WH, Greenberg RE, et al. **Hypoxia in human prostate carcinoma: an Eppendorf PO2 study. American journal of clinical oncology.** 2001;24(5):458-61. Epub 2001/10/05.

3.Helmlinger G, Yuan F, Dellian M, Jain RK. **Interstitial pH and pO2 gradients in solid tumors in vivo: high-resolution measurements reveal a lack of correlation. Nature medicine.** 1997;3(2):177-82. Epub 1997/02/01.

4.Tannock IF, Rotin D. **Acid pH in tumors and its potential for therapeutic exploitation.** Cancer research. 1989;49(16):4373-84. Epub 1989/08/15.

5.Sonveaux P, Vegran F, Schroeder T, Wergin MC, Verrax J, Rabbani ZN, et al. **Targeting lactate-fueled respiration selectively kills hypoxic tumor cells in mice. The Journal of clinical investigation.** 2008;118(12):3930-42. Epub 2008/11/27.

6.Pinheiro C, Longatto-Filho A, Azevedo-Silva J, Casal M, Schmitt FC, Baltazar F. **Role of monocarboxylate transporters in human cancers: state of the art. Journal of bioenergetics and biomembranes.** 2012;44(1):127-39. Epub 2012/03/13.

7.Halestrap AP, Wilson MC. **The monocarboxylate transporter family--role and regulation.** IUBMB life. 2012;64(2):109-19. Epub 2011/12/14.

8.Busk M, Horsman MR, Kristjansen PE, van der Kogel AJ, Bussink J, Overgaard J. **Aerobic glycolysis in cancers: implications for the usability of oxygen-responsive genes and fluorodeoxyglucose-PET as markers of tissue hypoxia.** International journal of cancer Journal international du cancer. 2008;122(12):2726-34. Epub 2008/03/21.

9.Semenza GL. **Regulation of cancer cell metabolism by hypoxia-inducible factor 1.** Seminars in cancer biology. 2009;19(1):12-6. Epub 2008/12/31.

10.Potter CP, Harris AL. **Diagnostic, prognostic and therapeutic implications of carbonic anhydrases in cancer.** British journal of cancer. 2003;89(1):2-7. Epub 2003/07/03.

11.Zha S, Ferdinandusse S, Hicks JL, Denis S, Dunn TA, Wanders RJ, et al. **Peroxisomal branched chain fatty acid beta-oxidation pathway is upregulated in prostate cancer.** The Prostate. 2005;63(4):316-23. Epub 2004/12/16.

12.Liu Y. **Fatty acid oxidation is a dominant bioenergetic pathway in prostate cancer.** Prostate cancer and prostatic diseases. 2006;9(3):230-4. Epub 2006/05/10.

13.Pertega-Gomes N, Vizcaino JR, Miranda-Goncalves V, Pinheiro C, Silva J, Pereira H, et al. **Monocarboxylate transporter 4 (MCT4) and CD147 overexpression is associated with poor prognosis in prostate cancer.** BMC cancer. 2011;11:312. Epub 2011/07/27.

14.Condon MS. **The role of the stromal microenvironment in prostate cancer. Seminars in cancer biology.** 2005;15(2):132-7. Epub 2005/01/18.

15.Yang F, Tuxhorn JA, Ressler SJ, McAlhany SJ, Dang TD, Rowley DR. S**tromal expression of connective tissue growth factor promotes angiogenesis and prostate cancer tumorigenesis.** Cancer research. 2005;65(19):8887-95. Epub 2005/10/06.

16.Arnold JT, Gray NE, Jacobowitz K, Viswanathan L, Cheung PW, McFann KK, et al. **Human prostate stromal cells stimulate increased PSA production in DHEA-treated prostate cancer epithelial cells.** The Journal of steroid biochemistry and molecular biology. 2008;111(3-5):240-6. Epub 2008/07/16.

17.Morrissey C, Vessella RL. **The role of tumor microenvironment in prostate cancer bone metastasis.** Journal of cellular biochemistry. 2007;101(4):873-86. Epub 2007/03/28.

18.Fiaschi T, Marini A, Giannoni E, Taddei ML, Gandellini P, De Donatis A, et al. **Reciprocal metabolic reprogramming through lactate shuttle coordinately influences tumor-stroma interplay.** Cancer research. 2012;72(19):5130-40. Epub 2012/08/02.

19.Giatromanolaki A, Koukourakis MI, Koutsopoulos A, Mendrinos S, Sivridis E. **The metabolic interactions between tumor cells and tumor-associated stroma (TAS) in prostatic cancer.** Cancer biology & therapy. 2012;13(13):1284-9. Epub 2012/08/17.

Figure 1

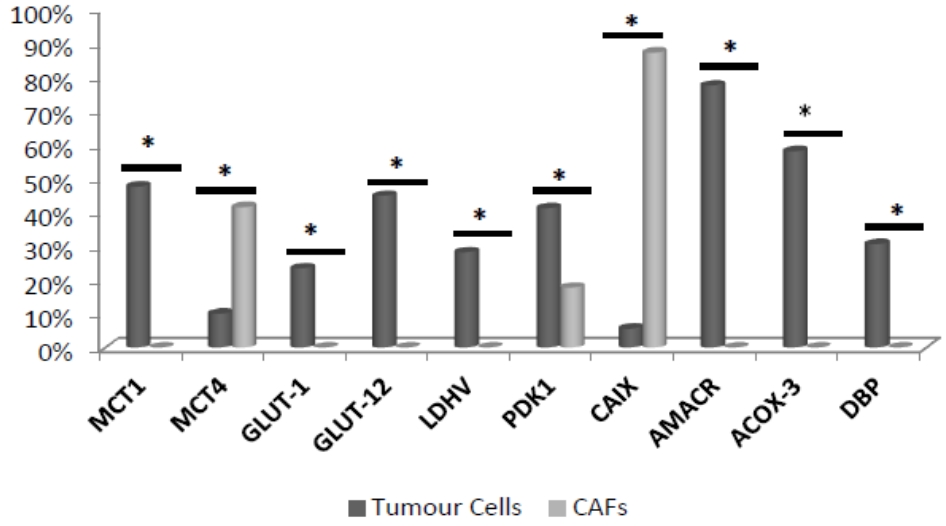

Figure 1. Graphic showing the comparison between metabolic-related proteins expression in Tumour Cells and cancer associated fibroblasts (CAFs).

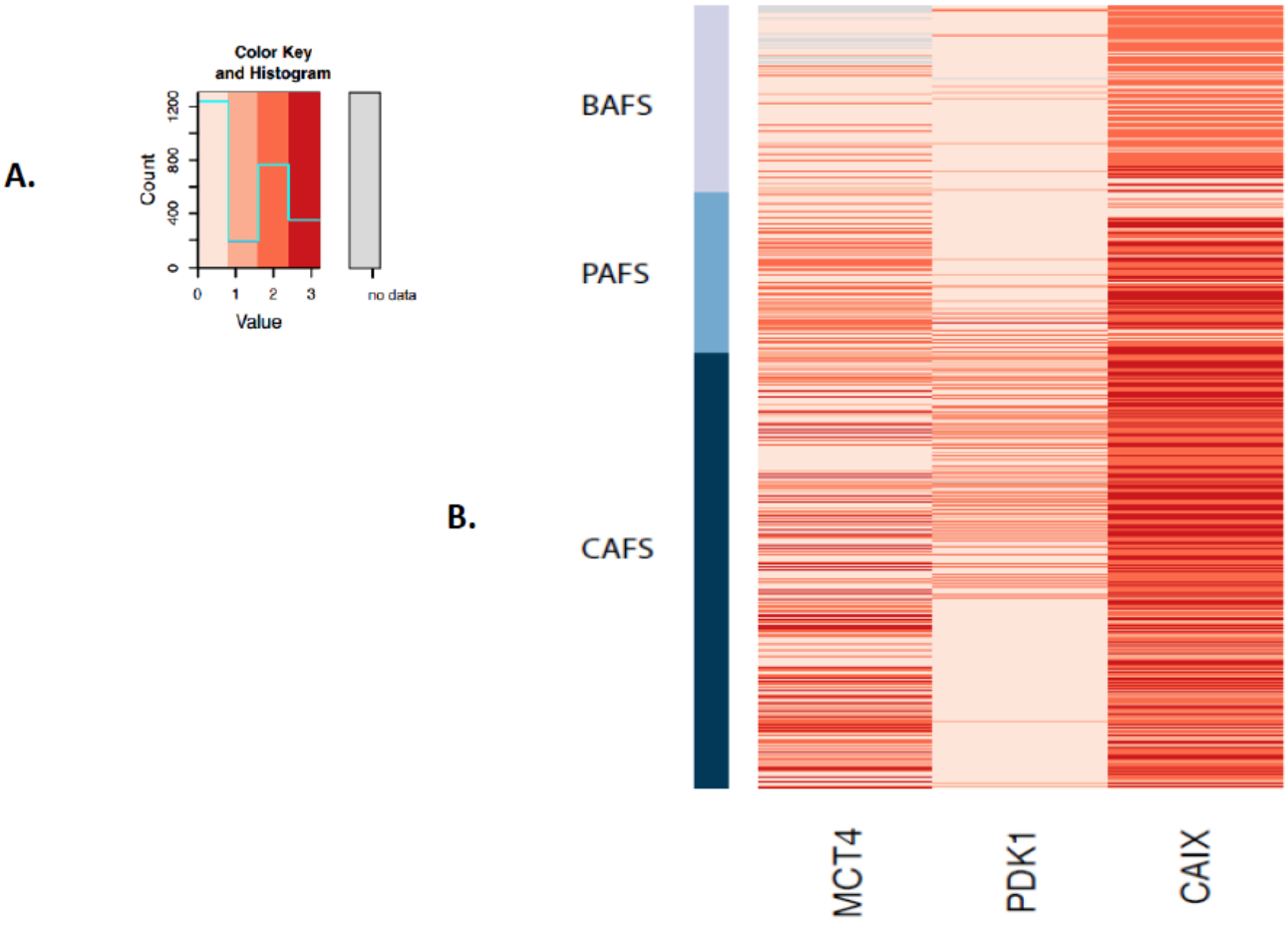

Figure 2. (A) Color code showing the codification of the staining score for each case represented in the heatmap: no staining (0), weak staining (1), moderate (2) and strong (3) staining are represented. (B) Heatmap representing MCT4, PDK1 and CAIX protein expression in benign glands associated fibroblasts (BAFs), PIN associated fibroblasts (PAFs) and cancer associated fibroblasts (CAFs), showing evident variations in the expression of these proteins.

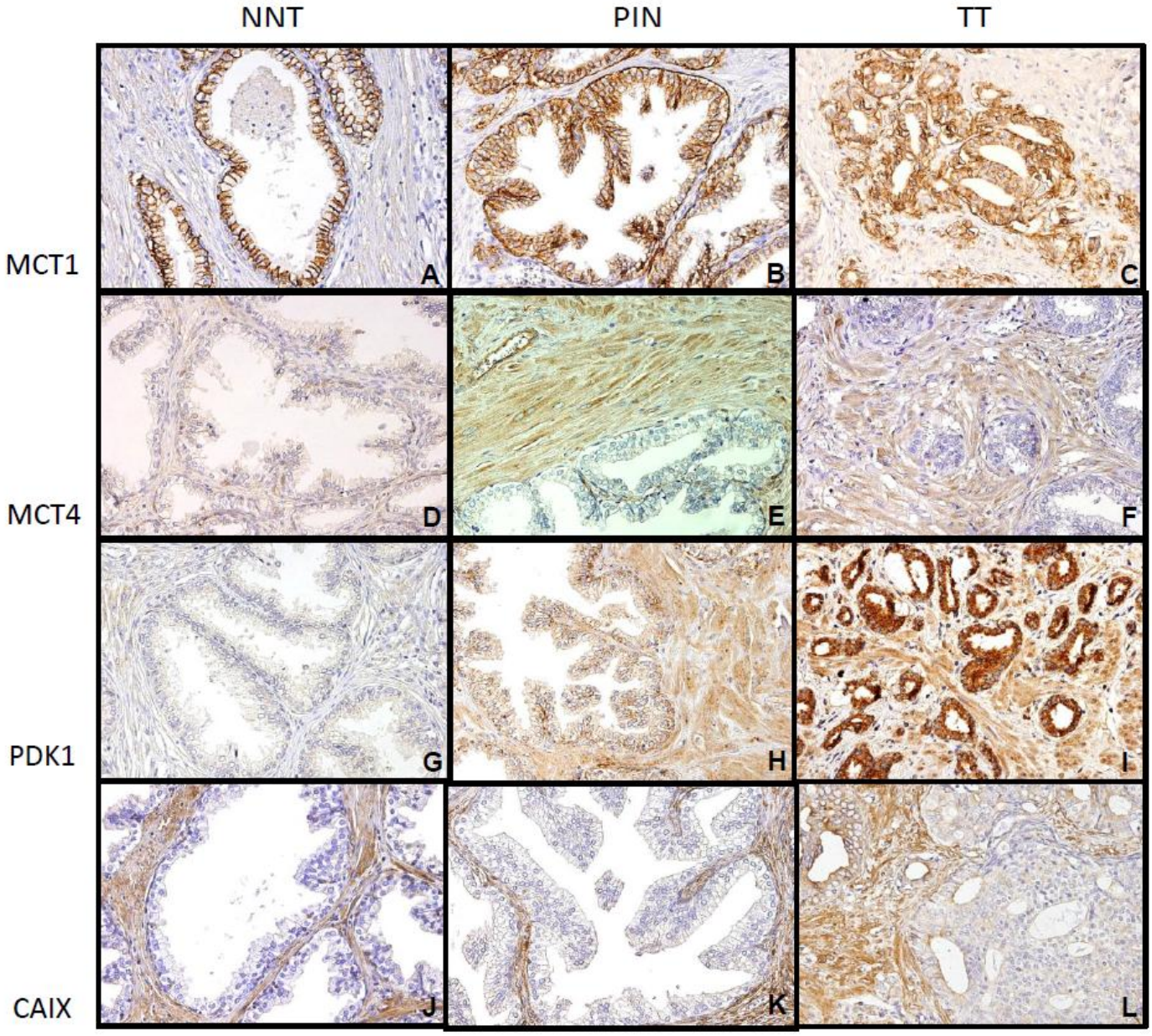

Figure 3. Immunohistochemical staining for MCT1, MCT4, PDK1 and CAIX expression in non-neoplastic tissue (NNT) , PIN lesions (PIN), tumour tissue (TT) and the surrounding stroma for each case. It is possible to observe the strong expression of MCT4, PDK1 and CAIX in stromal cells in contrast to MCT1 which was exclusively present in the epithelial cells of the glands.

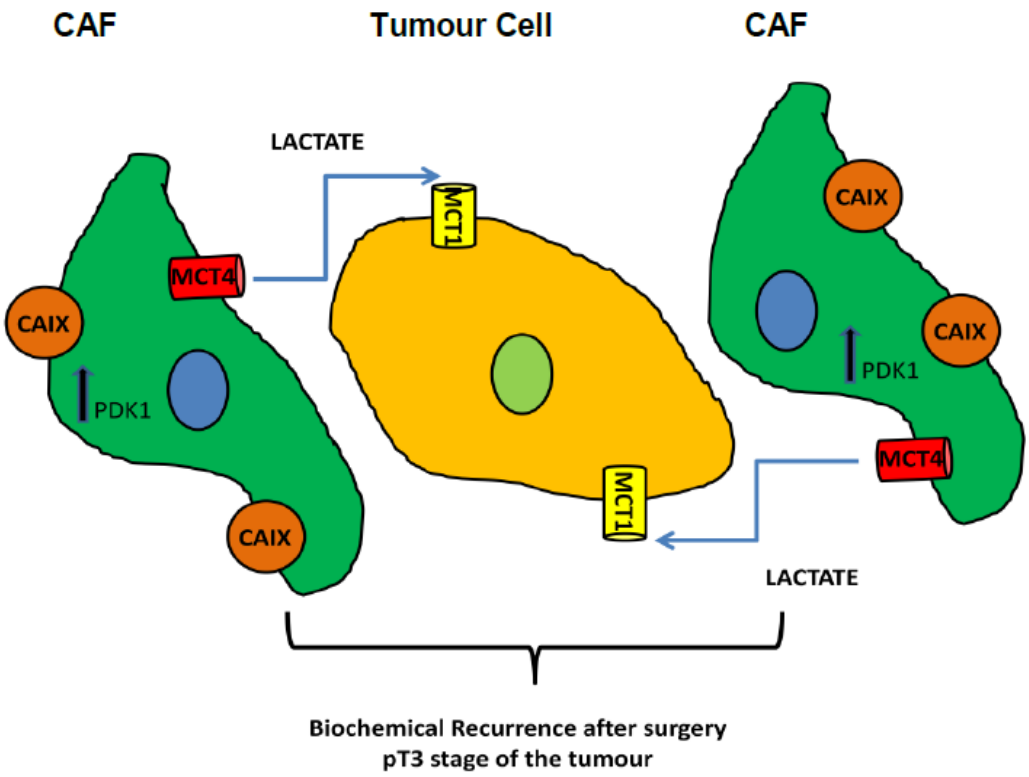

Figure 4. Schematic representation of the lactate shuttle system between malignant cells and cancer associated fibroblasts (CAFs) that correlate with poor prognosis. The expression of MCT4 in CAFs together with the expression of MCT1 in tumour cells are linked to the presence of biochemical recurrence after surgery and pT3 stage of the tumour.

Tables

Table 1. Details of the immunohistochemical procedure used to analyze the expression of the different proteins.

Protein	Antibody	Company	Antibody Dilution	Positive Control	Incubation Period	Detection System
MCT4	sc-50329	Santa Cruz Biotechnology	1:500	Colon tumor	Overnight	R.T.U. Vectastain Universal Elite ABC Kit, Vector, EUA
GLUT1	ab 15309	Abcam	1:2000	Head and neck tumor	2 hours	Ultravision Detection System Anti-polyvalent, HRP, Labvision Corporation, Freemont, CA
CAIX	ab 15086	Abcam	1:2000	Stomach		
MCT1	sc-365501	Santa Cruz Biotechnology	1:500	Colon tumor	Overnight	R.T.U. Vectastain Universal Elite ABC Kit, Vector, EUA
GLUT12	ab 75441	Abcam	1:500	Rim	Overnight	Ultravision Detection System Anti-polyvalent, HRP, Labvision Corporation, Freemont, CA
LDHV	ab 53010	Abcam	1:1000	Colon tumor	2 hours	
PDK1	ab110025	Abcam	1:500	Stomach		
AMACR	504R-16	Cell Marque	1:50	Kidney		
ACOX3	sc-135435	Santa Cruz Biotechnology	1:250	Liver		
DBP	DBP antibody was a gift from Dr. Gabriele Moller from HelmholtzZentrum mÜnchen.		Ready to use	Kidney		

Table 2. Correlations between the key metabolic-related proteins MCT4, PDK1 and CAIX expressions in CAFs and clinico-pathological data. The correlation between MCT1/MCT4 expression (MCT1 expression in prostate tumour cells with concomitant expression of MCT4 in CAFs) and the clinico-pathological data is also represented.

		MCT4		PDK1		CAIX		MCT1/MCT4	
Variable	**n**	%	***p***	%	***p***	%	***p***	%	***p***
PSA (ng/ml)			0.296		0.451		0.250		0.525
≤ 5.0	245	47.9		16.2		85.9		27.1	
>5.0	123	44.0		17.5		82.2		27.5	
pT			0.445		0.258		0.522		**0.009**
2	359	40.2		18.7		86.4		20.8	
3	99	41.7		15.2		86.9		33.3	
Perineural Invasion			0.155		**0.069**		0.399		0.255
Absent	119	45.4		13.4		85.8		26.9	
Present	327	39.4		19.8		87.2		23.3	
Biochemical Recurrence			0.104		0.176		**0.003**		**0.052**
Absent	410	40.2		18.5		75.4		22.6	
Present	69	49.3		13.0		89.0		32.8	

Chapter 6. Further insights on MCTs regulation across prostate cancer progression

6.1 Chapter Overview

In the previous chapters we show results suggesting an important involvement of MCTs in prostate cancer at a diagnostic, prognostic and therapeutic level. However, which mechanisms are involved on MCTs regulation across malignant transformation is still unknown. In this chapter we investigated MCTs expression at mRNA level in different *in vitro* models of prostate cancer progression and explore the potential cross-talk between Androgen Receptor (AR), the main driver of prostate cancer cell growth, and the different MCTs isoforms as well as the potential cross-talk between the key regulator of cellular energy homeostasis AMPK and MCTs expression. Also the effect on MCTs expression was also assessed in this chapter.

The results presented in this chapter were obtained in collaboration with the Urological Research Laboratory, Cancer Research UK Cambridge Institute University of Cambridge Li Ka Shing Centre and still unpublished.

We show that MCT1 is expressed in all the models studied with no clear link between androgen stimulation/inhibition, AMPK stimulation/inhibition or hypoxia. In contrast, MCT2 was shown to be present at very low levels in prostate cancer cell lines and clearly downregulated by androgens with an apparent link with AMPK stimulation and no relation with hypoxia. Importantly, MCT4 appears to be strongly correlated with an aggressive phenotype, increasing its expression according to disease aggressiveness and although independent of androgen stimulation or inhibition, is highly dependent on the environmental conditions, being highly stimulated by hypoxic conditions and so a suitable target for cells under hypoxia.

These results indicate that different MCT isoforms are differentially regulated across prostate malignant transformation, indicating that major drivers of these isoforms are distinct and so their identification in more detail is needed.

6.2 UNPUBLISHED RESULTS

The results presented in this chapter were obtained in collaboration with the Urological Research Laboratory, Cancer Research UK Cambridge Institute University of Cambridge Li Ka Shing Centre. These results are considered preliminary data on the MCTs regulation in prostate cancer and are currently being studied more deeply.

6.2.1 Monocarboxylate Transporters (MCTs) regulation during prostate cancer progression

Abstract

The androgen receptor (AR) is a key regulator of prostate growth and the principal drug target for the treatment of prostate cancer. Recently, AMP-activated protein kinase (AMPK) emerged as a potential target for cancer cell therapy due to the observation that activation of AMPK inhibits tumour cell growth. On the other hand, we have the microenvironmental conditions, namely hypoxia which is known to increase the expression of genes with a crucial role in tumours progression and aggressiveness. However, how MCTs expression is affected by these different major drivers in prostate cancer is largely unknown.

The aim of this work was to investigate MCTs expression at the mRNA level in different models of prostate cancer and their regulation during cancer progression and how they are affected by stimulation/inhibition of these major drivers of cancer progression.

Our results showed that MCT2 was shown to be present at very low levels in prostate cancer cell lines and clearly downregulated by androgens with an apparent link with AMPK stimulation using metformin. Importantly, MCT4 appears to be strongly correlated with an aggressive phenotype, increasing according to disease aggressiveness and although independent of androgen stimulation or inhibition is highly dependent on the environmental conditions, being highly stimulated by hypoxic conditions and so a suitable target for cells under hypoxia. In contrast, MCT1 was shown to increase under hypoxic conditions but only in LNCaP cells. These results show that different MCT isoforms are differentially expressed during prostate cancer progression and that they are regulated in different ways. We believe that these changes reflect the metabolic demands of prostate cancer tumours during disease progression in which MCTs play an important role. These results also indicate that more accurate information into the metabolic variations across malignant transformation may help in the risk stratification of patients, with further implications in the management of the disease.

Introduction

The androgen receptor (AR) is the main therapeutic target in prostate cancer. Androgen deprivation therapy (chemical castration) is an effective first-line therapy for prostate cancer, but despite good initial responses the recurrence of castrate-resistant disease is common and ultimately fatal. AR is essential for cell viability, proliferation and invasion in both hormone-sensitive and castrate-resistant prostate cancer [1-3]. Clinical studies supported these findings by reporting the sensitivity of castrate-resistant prostate cancer to second-generation AR antagonists and hormone synthesis blockade [4-6].

In castrate-resistant disease, where tumours are less sensitive to androgen depletion, AR activity is maintained by gene amplification [7], activating mutations [8,9] or signalling cross talk with other oncogenic pathways [10].

Massie CE *et al* [11] discovered that CAMKK2 is a key effector of the AR, regulating glycolytic flux by activating AMPK-PFK signalling, which in turn drives anabolism and thereby controls prostate cancer cell proliferation and tumour growth. However, the role of AMPK in prostate cancer is controversial [12,13]; however, AMPK has an essential energy sensing role in cells and is critical in determining cell fate under stress conditions [14]. Therefore, the timing, level and cellular context of AMPK activation may have profound effects on the functional consequences of AMPK signalling. For example, in normal somatic cells AMPK has been shown to activate glycolysis via PFK and inhibit protein biosynthesis by decreasing MTOR activity [15-16]. In contrast, Massie CE data indicates that CAMKK2 enhances metabolic flux by stimulating AMPK phosphorylation without any effect on mTOR activity [11]. The mechanisms underlying this skewing of AMPK signalling in this context are unclear; however, it is possible that other targets of the AR or CAMKK2 may impact on these downstream pathways.

Hypoxia is a common feature of prostate tumours, leading to increased gene instability, reduced treatment response, and increased tumour aggressiveness. Tumour hypoxia is progressively emerging as a common feature of prostate tumours associated with poor prognosis. While the molecular basis of disease

progression is increasingly well documented, the potential role of hypoxia in these processes remains poorly evaluated [17].

In this work we aim to study which mechanisms are involved in MCTs regulation in prostate cancer cells. For this purpose, we assessed the potential cross-talk between AR, the main driver of prostate cancer cell growth, and the different MCTs isoforms. Study of the potential cross-talk between the key regulator of cellular energy homeostasis AMPK (shown to regulate the activity of a number of transcription factors and involved in a different biochemical processes that might implicate the regulation of lactate production) and MCTs expression. Finally, we aimed to assess the effect of tumour microenvironment conditions, namely hypoxia on MCTs expression.

Materials and Methods

Cell lines and culture conditions

The human prostate cancer cell lines 22RV1, LNCaP, C4-2,C4-2B,PC3, DU145 as well as the non-transformed prostate cell line PNT1a were obtained form America Type Culture collection.Cell lines were maintained in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum. Androgen treatments were carried out in phenol-red free RPMI 1640 (Invitrogen) supplemented with 10% of charcoal/dextrane treated FBS) (Hyclone).

Drug treatments

AICAR (Tocris Bioscience) and metformin (Sigma) were dissolved in sterile PBS and used at final concentrations of 0.5 mM and 2 mM, respectively. Compound C (Tocris Bioscience) was dissolved in DMSO and used at 2uM, final concentration. R1881 and bicalutamide (Sigma) were used at concentrations of 1nM and 10uM, respectively.

Results

Monocarboxylate Transporters 1, 2 and 4 expression in prostate cancer cell lines

The expression pattern of MCTs isoforms were determined in a panel of prostate cell lines representing *in vitro* models of disease progression (Figure 1). PNT1a (non-transformed prostate), castrate sensitive (LNCaP) and castrate-resistant (C4-2 and C4-2B) as well as AR negative prostate cancer cell lines (PC3 and DU145) were used.

The obtained results show that mRNA levels for all MCTs were readily detectable by qrt-PCR in all 6 cell lines. It was interesting to observe that while MCT1 (Figure 1A) and MCT2 (Figure 1B) expression changed in a similar way across the cell lines studied, MCT4 mRNA levels clearly increases according to the aggressiveness of the prostate cancer cell models (Figure 1C). This first observation immediately suggests that different isoforms are being differently recruited according to the cell model/stage of the disease.

Effect of Androgen stimulation and inhibition on MCTs expression in prostate cell lines

Being AR an important regulator of metabolic pathways in prostate cancer cell lines, regulating the expression of various genes involved in metabolism [11] we aimed to elucidate the potential cross-regulation between MCTs and AR signaling. With this purpose, cells were treated with R1881, a potent, non-aromatizable androgen which has also been used as a photoaffinity label for the androgen receptor and Bicalutamide a non-steroidal anti-androgen used in the treatment of prostate cancer and the mRNA expression levels for the different MCTs were assessed during different times. To account for possible differences between castrate-sensitive and castrate-resistant cells, these experiments were performed in both LNCaP and C4-2B cells.

The results show that MCT2 clearly goes down with androgen treatment in both LNCaP and C4-2B cell lines. However, for MCT1 and MCT4 the effect of androgen

stimulation or inhibition was not so clear. Initially in both cell lines MCT1 seems to go down but at 48H an increase occurs in LNCaP cells. In contrast, MCT4 behaves in a total different and opposite way in LNCaP and C4-2B. These results point to the idea that these isoforms (MCT1 and MCT4) might have another major driven mechanism, being not directly dependent on androgen stimulation/inhibition.

These results were further confirmed looking at the available dataset from the group on LNCaP cell lines, showing that MCT2 expression definitely goes down with androgens (Figure 3). Also, AR binding sites were found near the gene and near the adjacent gene LRIG3 (which also is down-regulated), suggesting some locus-wide effect. Again, there were no major changes for MCT1 and MCT4 until 24h of treatment, suggesting that AR is not the major regulator of these two isoforms.

Effect of manipulating AMPK pathway on MCTs expression in prostate cancer cell lines

Due to its role as key regulator of cellular energy homeostasis, AMPK has been largely explored in the cancer field. However, the role of AMPK stimulating drugs as enhancers or inhibitors of tumour growth is still controversial. The effect of AMPK on MCTs expression is largely unknown, however there are some studies reporting that AICAR, is a cell permeable activator of AMPK treatment decreases MCT1 and increased MCT4 mRNA levels in rat Sertoli cells [18]. Also, another study suggests that AMPK stimulation increases both MCT1 and MCT4 protein expression in mice plantaris muscle [19], however the link between AMPK and MCTs expression in the cancer context is not understood.

Figure 4 shows the relative mRNA levels of MCTs when AMPK was stimulated by AICAR or Metformin and inhibited using Compound C in different *in vitro* models of prostate cancer and in the non-malignant cell line PNT1a. The results show that AICAR decreases MCTs mRNA levels in all the cell lines studied whereas in contrast Metformin has clearly the opposite effect on MCT2 mRNA levels. Regarding Compound C, the effects were not clear. It is shown to cause the opposite effect of AICAR and MTF on MCTs expression; however in most of the

cases Compound C maintains MCTs expression in the same levels as for untreated conditions (UT).

The only striking results taking from this experiment was the clear effect of MTF on MCT2 expression. This result was quite interesting if we think that MCT2 has been downregulated by androgens.

Again, the complexity of AMPK regulatory mechanisms is evident and also the doses of stimulators and inhibitors could cause different effects, as so, a further effort should be made in order to prove a possible link between AMPK and lactate transporters in prostate cancer.

The Effect of Hypoxic microenvironment on MCTs expression in prostate cancer cell lines

Tumour microenvironment is exposed to hypoxia which is one of the most recognized reasons for altered tumour metabolism. Perhaps the most important aspect of how cells respond to this unique microenvironment is the activity of the hypoxia-inducible factor 1 (HIF1) transcription factor. HIF has been massively explored in the cancer context and the link between HIF/hypoxia and MCTs has been already suggested, however in prostate cancer this correlation is not described. Figure 5 shows the expression levels of MCTs in LNCaP and C4-2B cell lines when grown in hypoxic conditions compared to normal oxygen levels.

The results clearly show a great increase in MCT4 expression under hypoxic conditions compared to normoxia in both cell lines. MCT1 also seems to increase in LNCaP cells under hypoxia whereas for MCT2 no clear changes were observed under low oxygen conditions.

Discussion:

Our previous data so far suggest a metabolic switch from less aggressive *in situ* tumor to highly aggressive and metastatic prostate tumor, which seems to rely more on glycolytic metabolism. Also, these results suggest important ramifications

in the prostate cancer treatment where MCTs could represent suitable targets in different stages of the disease and together with other metabolic related proteins represent a valuable tool at the prognostic level (Pértega-Gomes *et al, manuscript in preparation).* Although all the previous results point to a clear involvement of these transporters in prostate cancer, more work needs to be done in order to investigate and understand how MCTs are regulated and whether they represent promising targets for prostate cancer treatment in the *in vivo* context.

The results herein presented confirm the idea that different isoforms are differentially expressed across prostate malignant progression. Also it shows that they are driven by different regulatory mechanisms.

MCT4 mRNA levels clearly increase from LNCaP and C4-2 (low tumorigenic models) to C4-2B, PC3 and DU145 (highly tumorigenic models). Also, MCT4 expression clearly increases when the cells are under hypoxia and consequently this protein was shown to be a possible target for prostate cancer cells that are under hypoxic conditions. These results are in accordance with our previous speculations for MCT4 involvement in malignant progression and aggressiveness. In contrast, MCT1 only showed a clear response under hypoxic conditions in the androgen dependent model LNCaP and no clear link with androgens or AMPK stimulation/inhibition was observed. MCT2 expression was shown to be independent of oxygen conditions; however it was androgen dependent, decreasing under androgen stimulation and increasing under androgen blockage. Another curious observation regarding MCT2 expression was that it increases when cells were treated with metformin. In the future, the molecular alterations that underlie MCT1 and MCT2 expression will be subject of further study, however these first observations already allow us to say that a complex regulatory mechanism is behind MCTs expression in PCa, indicating that different isoforms are being differently recruited across disease progression.

References:

1. Haag P, Bektic J, Bartsch G, Klocker H, Eder IE (2005) Androgen receptor down regulation by small interference RNA induces cell growth inhibition in androgen sensitive as well as in androgen independent prostate cancer cells. J Steroid Biochem Mol Biol 96: 251–258.
2. Hara T, Miyazaki H, Lee A, Tran CP, Reiter RE (2008) Androgen receptor and invasion in prostate cancer. Cancer Res 68: 1128–1135.
3. Snoek R, Cheng H, Margiotti K, Wafa LA, Wong CA, Wong EC, Fazli L, Nelson CC, Gleave ME, Rennie PS (2009) *In vivo* knockdown of the androgen receptor results in growth inhibition and regression of well-established, castration-resistant prostate tumors. Clin Cancer Res 15: 39–47.
4. Attar RM, Jure-Kunkel M, Balog A, Cvijic ME, Dell-John J, Rizzo CA, Schweizer L, Spires TE, Platero JS, Obermeier M, Shan W, Salvati ME, Foster WR, Dinchuk J, Chen SJ, Vite G, Kramer R, Gottardis MM (2009) Discovery of BMS-641988, a novel and potent inhibitor of androgen receptor signaling for the treatment of prostate cancer. Cancer Res 69: 6522–6530.
5. Attard G, Reid AH, A'Hern R, Parker C, Oommen NB, Folkerd E, Messiou C, Molife LR, Maier G, Thompson E, Olmos D, Sinha R, Lee G, Dowsett M, Kaye SB, Dearnaley D, Kheoh T, Molina A, de Bono JS (2009) Selective inhibition of CYP17 with abiraterone acetate is highly active in the treatment of castration-resistant prostate cancer. J Clin Oncol 27: 3742–3748.
6. Tran C, Ouk S, Clegg NJ, Chen Y, Watson PA, Arora V, Wongvipat J, Smith-Jones PM, Yoo D, Kwon A, Wasielewska T, Welsbie D, Chen CD, Higano CS, Beer TM, Hung DT, Scher HI, Jung ME, Sawyers CL (2009) Development of a second-generation antiandrogen for treatment of advanced prostate cancer. Science (New York, NY) 324: 787–790.
7. Visakorpi T, Hyytinen E, Koivisto P, Tanner M, Keinanen R, Palmberg C, Palotie A, Tammela T, Isola J, Kallioniemi OP (1995) *In vivo* amplification of the androgen receptor gene and progression of human prostate cancer. Nat Genet 9: 401–406.
8. Veldscholte J, Ris-Stalpers C, Kuiper GG, Jenster G, Berrevoets C, Claassen E, van Rooij HC, Trapman J, Brinkmann AO, Mulder E (1990) A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens. Biochem Biophys Res Commun 173: 534–540
9. Steinkamp MP, O'Mahony OA, Brogley M, Rehman H, Lapensee EW, Dhanasekaran S, Hofer MD, Kuefer R, Chinnaiyan A, Rubin MA, Pienta KJ, Robins DM (2009) Treatment-dependent androgen receptor mutations in prostate cancer exploit multiple mechanisms to evade therapy. Cancer Res 69: 4434–4442.
10. Craft N, Shostak Y, Carey M, Sawyers CL (1999) A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase. Nat Med 5: 280–285.

11. Massie CE, Lynch A, Ramos-Montoya A, Boren J, Stark R, Fazli L, Warren A, Scott H, Madhu B, Sharma N, Bon H, Zecchini V, Smith DM, Denicola GM, Mathews N, Osborne M, Hadfield J, Macarthur S, Adryan B, Lyons SK, Brindle KM, Griffiths J, Gleave ME, Rennie PS, Neal DE, Mills IG. (2011). The androgen receptor fuels prostate cancer by regulating central metabolism and biosynthesis. EMBO J. 2011 May 20;30(13):2719-33.
12. Park HU, Suy S, Danner M, Dailey V, Zhang Y, Li H, Hyduke DR, Collins BT, Gagnon G, Kallakury B, Kumar D, Brown ML, Fornace A, Dritschilo A, Collins SP (2009) AMP-activated protein kinase promotes human prostate cancer cell growth and survival. Mol Cancer Ther 8: 733–741.
13. Zhou J, Huang W, Tao R, Ibaragi S, Lan F, Ido Y, Wu X, Alekseyev YO, Lenburg ME, Hu GF, Luo Z (2009) Inactivation of AMPK alters gene expression and promotes growth of prostate cancer cells. Oncogene 28: 1993–2002.
14. Liang J, Shao SH, Xu ZX, Hennessy B, Ding Z, Larrea M, Kondo S, Dumont DJ, Gutterman JU, Walker CL, Slingerland JM, Mills GB (2007) The energy sensing LKB1-AMPK pathway regulates p27(kip1) phosphorylation mediating the decision to enter autophagy or apoptosis. Nat Cell Biol 9: 218–224.
15. Marsin AS, Bertrand L, Rider MH, Deprez J, Beauloye C, Vincent MF, Van den Berghe G, Carling D, Hue L (2000) Phosphorylation and activation of heart PFK-2 by AMPK has a role in the stimulation of glycolysis during ischaemia. Curr Biol 10: 1247–1255.
16. Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, Turk BE, Shaw RJ (2008) AMPK phosphorylation of raptor mediates a metabolic checkpoint. Mol Cell 30: 214–226.
17. Marignol L., Rivera-Figueroa K., Lynch T. and Hollywood D. Hypoxia, notch signalling, and prostate cancer. Nature Reviews Urology 10, 405-413 (July 2013) doi:10.1038/nrurol.2013.110.
18. Galardo, M.N., et al., *The AMP-activated protein kinase activator, 5-aminoimidazole-4-carboxamide-1-b-D-ribonucleoside, regulates lactate production in rat Sertoli cells.* J Mol Endocrinol, 2007. **39**(4): p. 279-88.
19. Bonen, A., et al., *Isoform-specific regulation of the lactate transporters MCT1 and MCT4 by contractile activity.* Am J Physiol Endocrinol Metab, 2000. **279**(5): p. E1131-8.

Figures

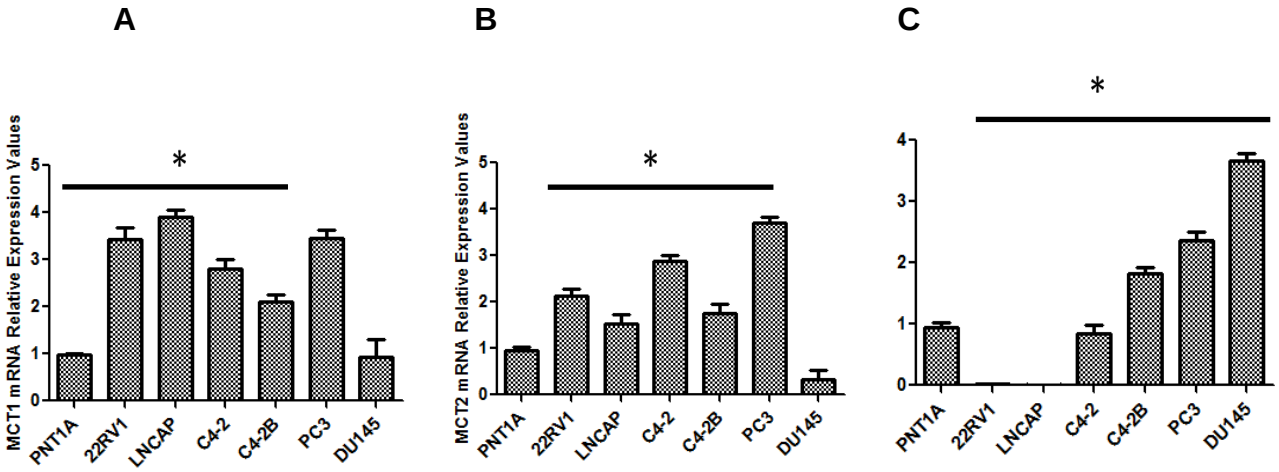

Figure 1. Expression levels of MCTs 1, 2 and 4 in prostate-derived cell lines. RNA was isolated from PNT1a, LNCaP, C4-2, c4-2B, PC-3 and DU145 cells. RNA levels of MCT1 (A), MCT2 (B) and MCT4 (C) were determined. For RNA quantification, n=3 ((*) indicates that the difference in MCTs expression between the non-malignant PNT1a and the malignant models is statistically significant ($p<0.005$)).

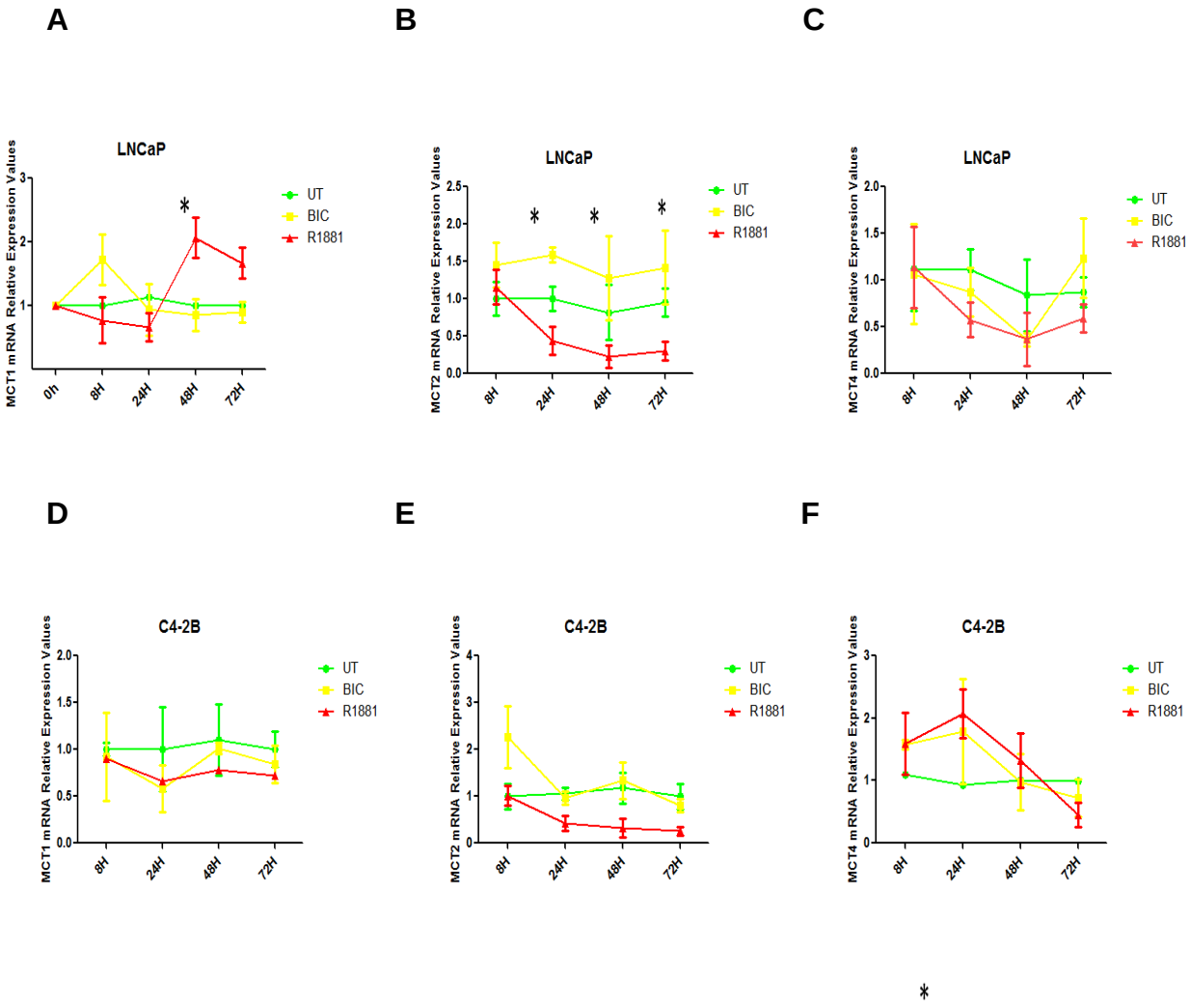

Figure 2: Effect of Androgen stimulation and inhibition on MCTs expression. Levels of MCT1 (A), MCT2 (B) and MCT4 (C) in LNCaP cell lines and levels of MCT1 (D), MCT2 (E) and MCT4 (F) in C4-2B cell lines after R1881 (R1881) and bicalutamide treatment (BIC) or with no treatment (UT) at different time-points are presented ((*) indicates that there is a statistically significant different between MCTs expression in the UT *vs* treated conditions ($p<0.005$)).

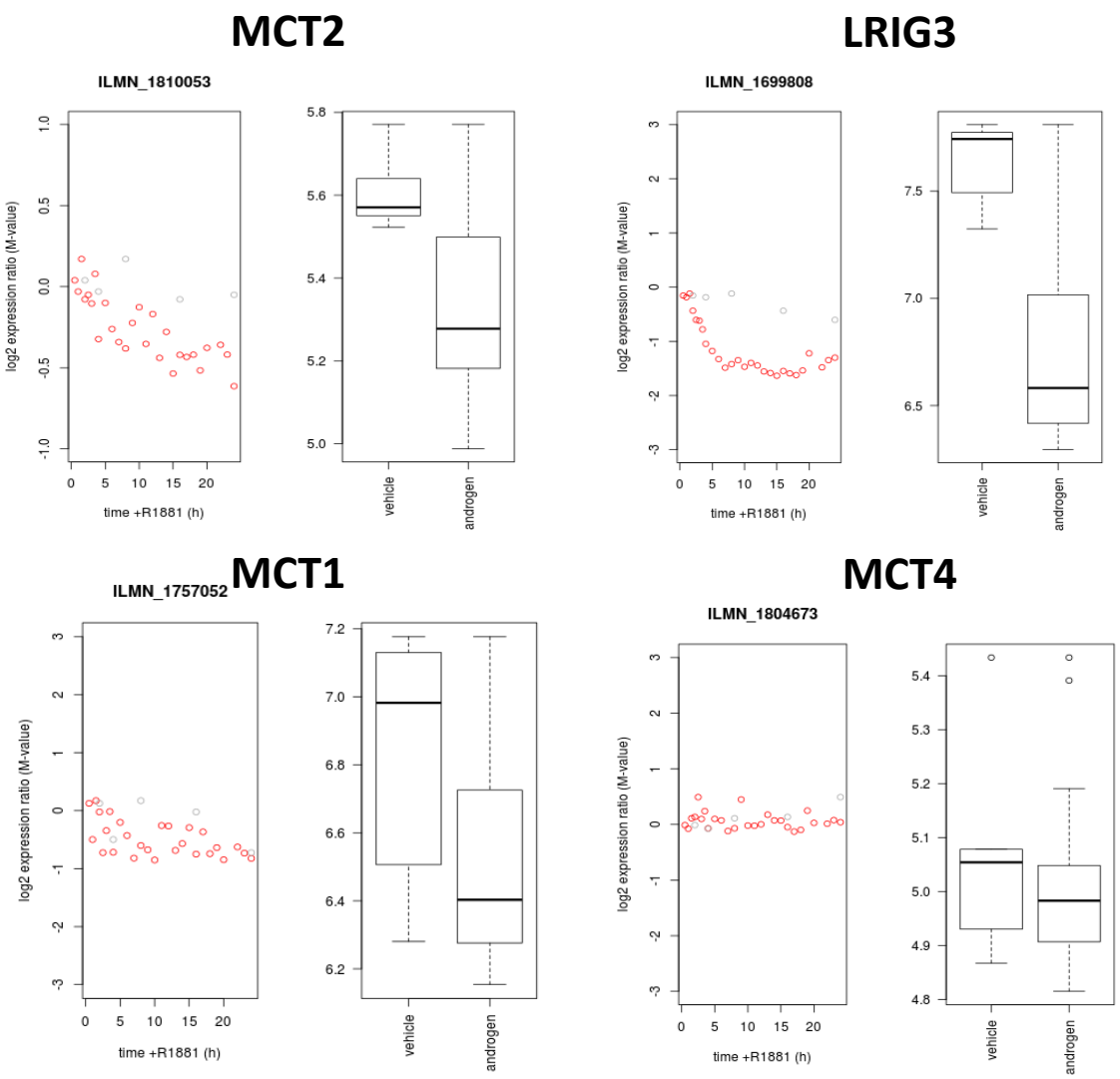

Figure 3. Plot from the expression array with androgen treatment time-course. (Massie C.)

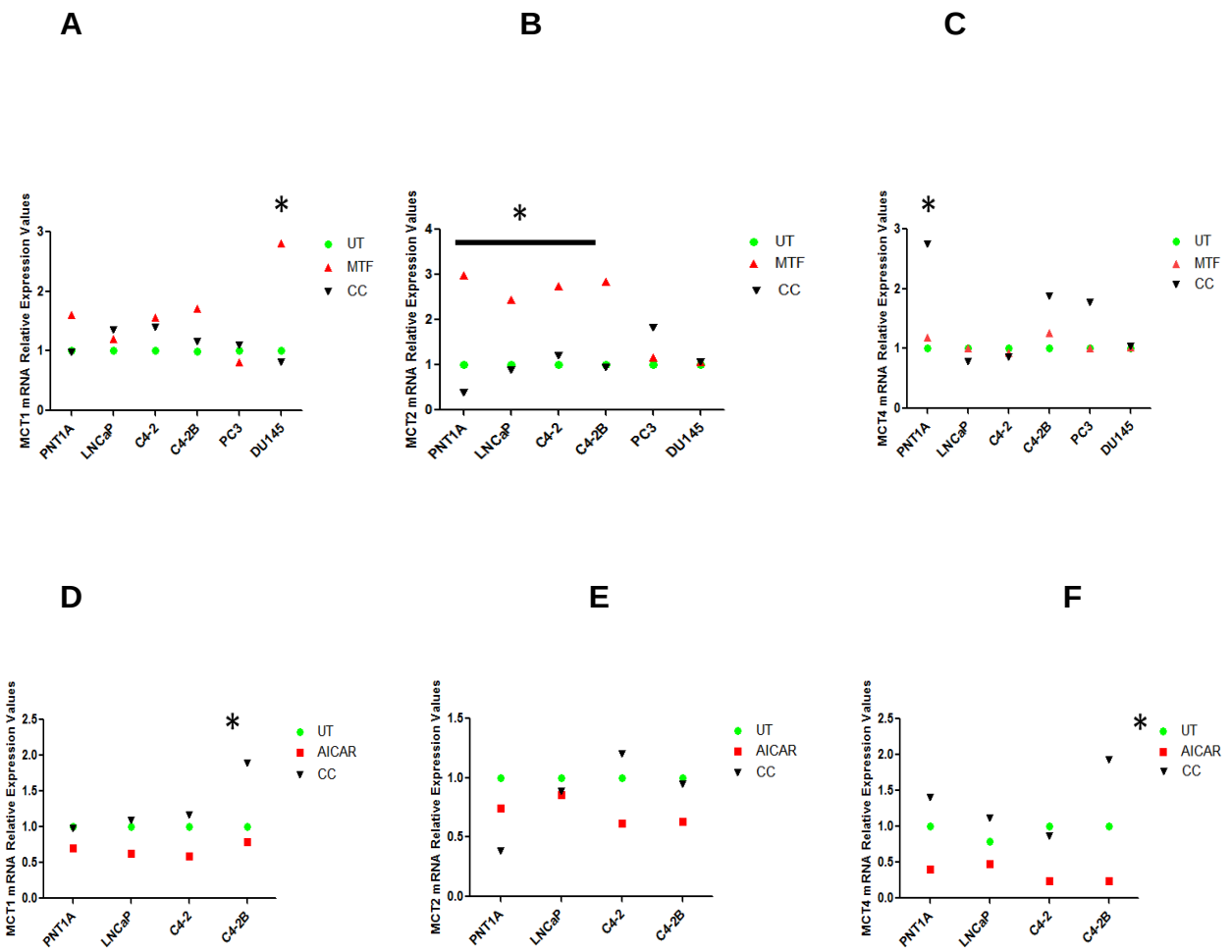

Figure 4: Effect of AMPK activity manipulation (by pharmacological activators and inhibitors) on MCTs expression. Levels of MCT1 (A), MCT2 (B) and MCT4 (C) in PNT1A, LNCaP, C4-2 and C4-2B cells untreated (UT) treated with 0.5mM AICAR (AICAR) or 2 uM CC (CC) for 48h; Levels of MCT1 (D), MCT2 (E) and MCT4 (F) in PNT1a, LNCaP, C4-2, C4-2B, PC3 and DU145 cells treated with 2mM of metformin or 2uM CC for 48h ((*) indicates that there is a statistically significant different between MCTs expression in the UT *vs* treated conditions ($p<0.005$)).

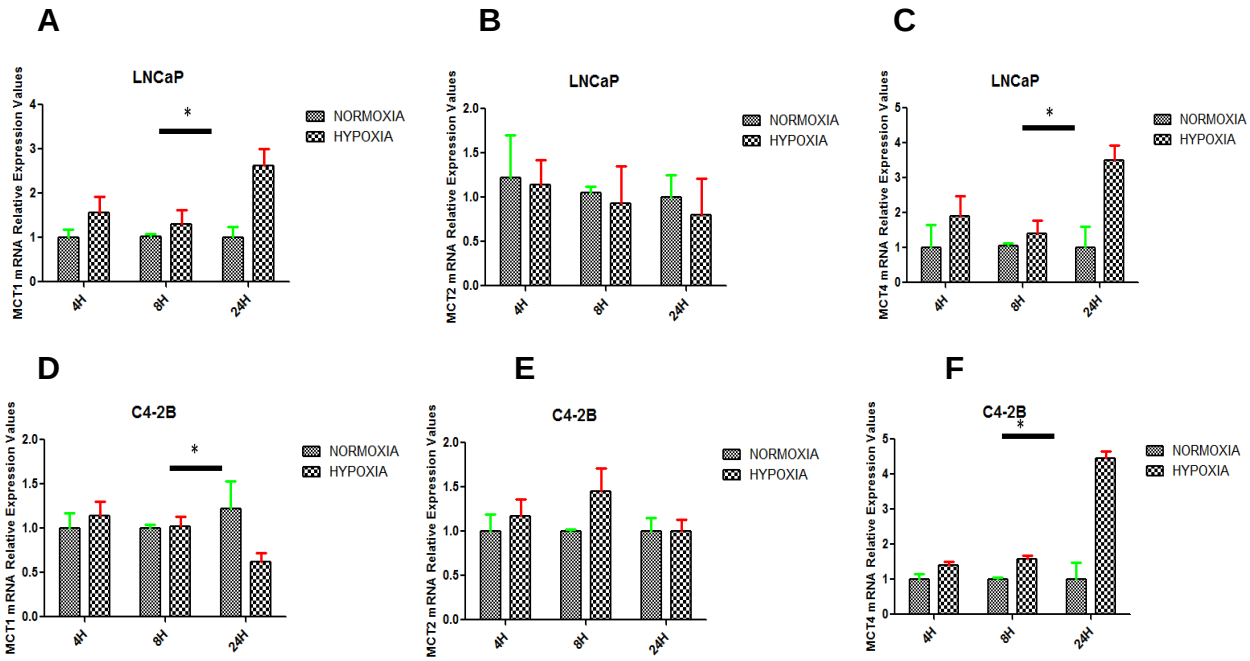

Figure 5: Effect of hypoxic conditions (1% O_2) on MCTs expression. Levels of MCT1 (A), MCT2 (B) and MCT4 (C) in LNCaPs under normoxic and hypoxic conditions ; Levels of MCT1 (D), MCT2 (E) and MCT4 (F) in C4-2B cells under normoxic and hypoxic conditions (*p<0.005).

CHAPTER 7. DISCUSSION AND FINAL CONSIDERATIONS

7.1 Discussion and Final Considerations

As state before, recent models of carcinogenesis integrate the neo-Darwinian evolution, stating that phenotypic properties are retained or lost based on their contribution for cells survival with cell-environment interactions. This new concept of carcinogenesis was applied to explain the preference of cancer cells for the glycolytic phenotype, even in the presence of oxygen (Warburg effect). Thus, as cancer progression proceeds, mutations in tumour cells increase and traits that are found in invasive cancers, like the hyperglycolytic and acid-resistant phenotypes, arise as adaptive mechanisms to environmental proliferative constraints, such as hypoxia. Many players have been involved in these cellular adaptations; however, and although an important role of lactate transporters could be expected in the context of the Warburg effect, the knowledge on the role of MCTs in solid tumours and their potential as targets in cancer therapy is still very scarce. Also, it is important to keep in mind that in order to be used as therapeutic targets in cancer, MCTs must be differently expressed, either in quantity or in isoform. Thus, there is an urgent need to determine MCTs behavior during the carcinogenesis process.

In this thesis, an important step forward was taken towards the possibility of exploiting MCTs as targets for prostate cancer therapy, accordingly to the stage of the disease. Firstly, a large series of human prostate tissue comprising benign tissue, PIN lesions, localized tumours and metastatic tissue was constructed. This series was well characterized and all the relevant clinico-pathological features were described and included in a detailed way in order to represent the heterogeneity of prostate cancer disease.

The study of MCTs and CD147 expression in this well characterized series revealed an important association between MCT4 and CD147 expression and reliable poor prognosis markers of disease progression namely Gleason score, pT3 stage of the tumour and biochemical recurrence after surgery. This first finding was of great importance as, the tumours that present these characteristics have a more aggressive clinical behavior and, until now, do not have a specific molecular therapy. Additionally, in the same study we found MCT2 to be highly

expressed in the cytoplasm of prostate cancer specimens and PIN lesions however with no clinico-pathological associations suggesting a role in malignant transformation more than in the disease aggressiveness.
Although we have found some important correlations, the presence of MCT1 in both tumour cells and non-malignant tissues as well as the absence of MCT4 at the plasma membrane lead us to hypothesize that prostate cancer might rely less than the majority of tumours on aerobic glycolysis.

Moving forward in order to test this hypothesis, we found that in contrast to the majority of tumours that rely on glycolytic metabolism and in which the so called "metabolic switch" occurs in the malignant transformation, in prostate cancer this switch seems to occur only in the progression to the metastatic disease. Only the highly tumorigenic cell lines showed high levels of glucose consumption and lactate production in contrast to the low tumorigenic models. Importantly, when proteins involved in aerobic glycolysis were expressed in localized tumour, they correlated with poor clinical outcome, indicating an immunophenotype with prognostic value.
The fact that prostate cancer might not fit in the typical model of high glycolytic transformation from benign to malignant glands, turned this work even more challenging in an attempt to contextualize the expression and consequently the role of different MCTs isoforms across prostate malignant transformation and progression. Being MCT2 highly expressed in localized tumour and MCT4 in the metastatic tissue this was indicative that the carcinogenic process leading to metastatic tumour, may require metabolic adaptations involving specific MCT upregulation and specific sub- cellular localization across different stages of the disease. Interestingly, MCT2 was found mainly in the localized disease with a high specificity and sensitivity to detect malignant glands, and its expression was shown to be similar to AMACR, a well established prostate cancer biomarker, being suggested as a putative prostate cancer biomarker, however, as well as AMACR, its expression was not correlated with poor prognosis parameters but it was present in high grade PIN (HGPIN) suggesting a possible role in the malignant transformation. Later, we found that this transporter co-localizes with the peroxisomal clusters and the formation of these clusters was observed from the non-malignant *in vitro* model PNT1a to 22RV1. The clusters as well as the co-

localization of MCT2 with peroxisomes decreased across the different models of disease aggressiveness, suggesting again a possible role for this protein in the malignant transformation rather than aggressiveness of the disease. It is important to note that MCT2 has been the less explored isoform, possibly due not only to the fact that only few tumours exhibit an high expression of this isoform but also because it has not been reported at the plasma membrane, turning it not part of the classical Warburg effect model but a transporter with other but maybe not less important functions that needs to be unveiled. It was curious to find that MCT2 mRNA levels consistently decrease with androgen stimulation, and increase with metformin, however these observations need to be further explored.

Of all the three isoforms, MCT4 was the one showing the most striking changes across malignant transformation. Not only MCT4 expression increases across the different *in vitro* models of disease progression but also its localization changes from cytoplasm to the plasma membrane. We showed that hypoxia might be the major driven mechanism underlying MCT4 expression across prostate progression and also that this isoform is a good target for cells under hypoxic conditions.

As stated before, MCT1 was expressed both in the tumour cells and *in vitro* models that exhibited more and less glycolytic behavior; its specific inhibition showed that MCT1 is a suitable target for cells either under normoxia or hypoxia. This indicates that MCT1, besides being important in oxidative cancer cells, may also be important in glycolytic cancer cells. In fact, the kinetic parameters of MCT1 make this isoform suitable for both the uptake and efflux of substrates. The role of MCT1 in both types of metabolic cancer cells makes it an even more interesting therapeutic target. It is not clear yet which mechanism drives MCT1, however we showed that hypoxia should have some influence since MCT1 mRNA levels increase in LNCaP under hypoxia, and in fact and since LNCaP was shown to be the cell line with less MCT4, MCT1 represents a good target for this model under hypoxia, however we should take into account that although MCT1 levels were higher in prostate cancer cells, normal prostate glands also express MCT1. Importantly, additional *in vitro* studies with MCT specific inhibition, evaluating parameters of aggressive behavior, such as migration, invasion and colony formation capacity will shed some light on the true value of MCTs. Indeed, MCT4

has already been associated with migration capacity. Of note, one should always remember that the main goal of MCT inhibition is the possible clinical application, so, the use of compounds that could have future clinical application, such as CHC and AstraZeneca compounds, ultimately offers a more advantageous approach.

Considering MCTs in the light of the microenvironmental model of carcinogenesis, where they shall have a vital role in the emergence of both the hyper-glycolytic and acid-resistant phenotypes by enabling lactate efflux from cancer cells, as well as regulate the intracellular pH and, the recent model of metabolic symbiosis between glycolytic and oxidative cancer cells, where lactate plays a key role as the metabolic intermediate, MCTs are essential players in this process. It is proposed in this model that lactate release from glycolytic/hypoxic fibroblasts occurs through the low-affinity lactate transporter MCT4 and lactate uptake by the oxidative/oxygenated cancer cells occurs through the high-affinity MCT1 transporter. Importantly, we explored for the first time the clinic-pathological significance of the establishment of this lactate shuttle observed that appeared to be linked to poor prognosis parameters namely the presence of biochemical recurrence after surgery, suggesting that immunohistochemical detection of proteins involved in the lactate shuttle may potentially prove to be useful as prognostic markers. In fact, and since we observed that CHC was able to decrease viability of prostate cancer cells but with no effect in glucose consumption or lactate production, corroborates the hypothesis that in prostate cancer the presence of MCT1 in the plasma membrane of cancer cells should be for the uptake of lactate instead of the efflux.

With this work, some contribution was also made to understand MCT regulation by chaperones. Firstly, the regulation of MCT1 and MCT4, but not MCT2, by CD147, was supported by evidence on human tissues. Importantly, there was undetected expression of gp70 in prostate cancer samples, suggesting that a not yet identified chaperone is involved in MCT2 trafficking. In this regard we found PEX19 interacting with MCT2, improving the knowledge on MCT2 regulation and function.

The data obtained so far points to the presence of different metabolic phenotypes across malignant transformation in which different isoforms of MCTs are differentially involved. As so, different isoforms of MCTs seem to be involved in different stages of prostate cancer progression. In one hand MCT1 and MCT2 seemed important in the maintenance of localized disease whereas MCT4 being independent of androgen stimulation and highly dependent on hypoxia is related with the aggressive phenotype, pointing to the idea that targeting the different MCTs across prostate cancer disease progression seems promising.

Much was achieved with this work but many other doors are now open that should be explored. Besides the more obvious lines that can be further explored, other directions can be taken, such as the study of other metabolic pathways like glutaminolysis, microenvironmental conditions like acidity and hypoxia, and other players in MCT regulation such as HIF-1a, AKT, c-myc and others. Importantly, since cell culture does not mimic all real tumour conditions, including O_2 and nutrient limitation, key factors in metabolism, it is fundamental to assess the effects of MCT inhibition in an animal model, in what concerns tumour survival and aggressiveness. Therefore, *in vivo* studies evaluating the effect of MCT inhibition in, among others, tumour growth, angiogenesis and metastization are essential. In parallel, as MCTs are also important in physiological homeostasis, toxicity studies to determine MCT inhibition side effects will determine the actual potential of MCTs as therapeutic targets in cancer.

In conclusion, the results herein presented encourage the exploitation of MCTs, as potential targets for prostate cancer therapy, and pave the way for further efforts to understand the role of MCTs in solid tumours that as prostate cancer might not rely mainly on glycolytic metabolism. Although major advances have been made with the present work, it elicits for many other studies to complement the present knowledge on MCTs' role in prostate tumour survival and aggressiveness.